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by

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Abstract

A mutation in *Saccharomyces cerevisiae* which affects the modification of uridine to dihydrouridine in tRNA molecules has been identified and characterized. The mutant tRNA phenotype was first reported in strain XB109-5B as a multiple isoacceptor profile for tRNATyr' by RPC-5 chromatography. The multiple isoacceptor tRNA profile was later extended to tRNAPhe, tRNASer and tRNAVal.

In this study, besides extending the multiple isoacceptor profiles to other tRNAs, a genetic basis for the production of these multiple isoaccepting tRNA profiles was established by analysing the tRNA profiles in the diploids as well as the progeny tetrads from a cross between XB109-5B and wild type (S288C). A single recessive mutation (designated *mia*) was implicated for the production of the multiple (mutant) tRNA profiles. The pleiotropic effects of *mia* were also studied in one of the progeny tetrads and the results indicate that *mia* is responsible for the production of the extra isoaccepting tRNAs in the six tRNA species that are known to exhibit a mutant tRNA profile.

The molecular lesion causing the production of the mutant isoaccepting tRNAs in *mia* was identified by nucleoside composition analyses of the purified tRNAPhe and one of the tRNATyr mutant isoacceptors. The results indicate that the mutant tRNAs are lacking some of the dihydrouridine moieties normally present.

' Through out this text, tRNAXXX=$tRNA^{XXX}$

Results from the aminoacylation experiments indicate that the mutant tRNAs are produced at the expense of the normal levels of wild type tRNAs. Studies on the production of these mutant tRNAs indicate that they are the predominant tRNA species at the logarithmic stages of a *mia* culture. These results suggest that the mutant tRNA isoacceptors in *mia* , although lacking some of the dihydrouridine modification(s), are functional in protein synthesis. This hypothesis is supported by the activities of the individual tRNAPhe isoacceptors in the *in vitro* poly-U translation experiments.

Data from *in vivo* pulse-label and chase experiments demonstrate that the mutant tRNAs can be converted to the cognate wild type molecules. This indicates that the mutant tRNAs may be precursor molecules accumulated due to a defective modification step and that they can still be processed to the wild type molecules. Results from the analysis of a *mia trm1* double mutant support the hypothesis that the maturation of tRNA does not necessarily follow an obligatory sequence.

The effect of lacking the dihydrouridine modification(s) on the function of tRNA was further examined by studying the efficiency of the ochre suppressor *SUP4* in the presence of *mia* . The results indicate that the activity of *SUP4* does not seem to be altered in spite of the dihydrouridine deficiency.

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I. Introduction

The existence of transfer RNA was predicted by Francis Crick in the mid 1950s as a type of adaptor molecule necessary for the transfer of information from DNA into proteins (Crick, 1955). He postulated at least 20 different kinds of adaptor molecule, corresponding to the 20 amino acids, and 20 different enzymes to link the amino acids to the appropriate adaptors. These adaptor molecules, together with the specific amino acids, would then combine specifically with the DNA template during the production of polypeptides. Within a few years, the tRNAs (then called soluble RNAs) and the aminoacyl-tRNA synthetases (ligases) were discovered (Hoagland *et al.*, 1957; Hoagland *et al.*, 1958; Berg and Ofengand, 1958). Early experiments were focused on the sequencing of different tRNA species (Holley *et al.*, 1965; Madison *et al.*, 1966; Zachau *et al.*, 1966; RajBhandary *et al.*, 1966) and the specificities of tRNAs during aminoacylation reactions (Söll *et al.*, 1966). The interactions of aminoacyl-tRNAs with particular oligonucleotides (Speyer *et al.*, 1963; Khorana *et al.*, 1966) were very important in deciphering the genetic code (Crick, 1966) as well as understanding the biological activity of tRNA during protein synthesis (Mclaughlin *et al.*, 1966); a function of tRNA predicted by Francis Crick before its discovery. Today, the role of tRNA in protein synthesis is

generally understood even though the details of interactions between aminoacyl-tRNAs, ribosomes, mRNA and other protein factors are still unclear (reviewed by Pongs, 1978).

Recently , data have accumulated which indicate the involvement of tRNA in other cellular functions (reviewed by LaRossa and Söll, 1978). Transfer RNAs have been implicated in the regulation of amino acid biosynthesis (reviewed by Umbarger, 1980; Eisenberg *et al.*, 1980), branched-chain amino acid transport (Quay and Oxender, 1980), transfer of amino acids to specific protein acceptors (Soffer, 1980) and as primers for reverse transcriptases (Dahlberg, 1980). It appears that tRNA is one of the most versatile molecules in the cell and it would not be surprising to discover more functions of tRNA with more studies.

The first tRNA sequence was determined in 1965 (Holley *et al.*, 1965); since then, over 120 different tRNA sequences from a variety of organisms have been reported (Gauss and Sprinzl, 1981). Some interesting features of tRNA are apparent by examining these sequences. All tRNA molecules are 75 to 93 nucleotides long, and can be arranged into a very similar secondary structure called the cloverleaf configuration (Holley *et al.*, 1965; Barrell and Clark, 1974; Clark ,1978). Several stem and loop regions can be recognized in this secondary structure which maximize Watson-Crick base pairing among the nucleotides (Fig. 1). The stem regions are very constant in terms of the number of base paired nucleotides while the loop regions are variable

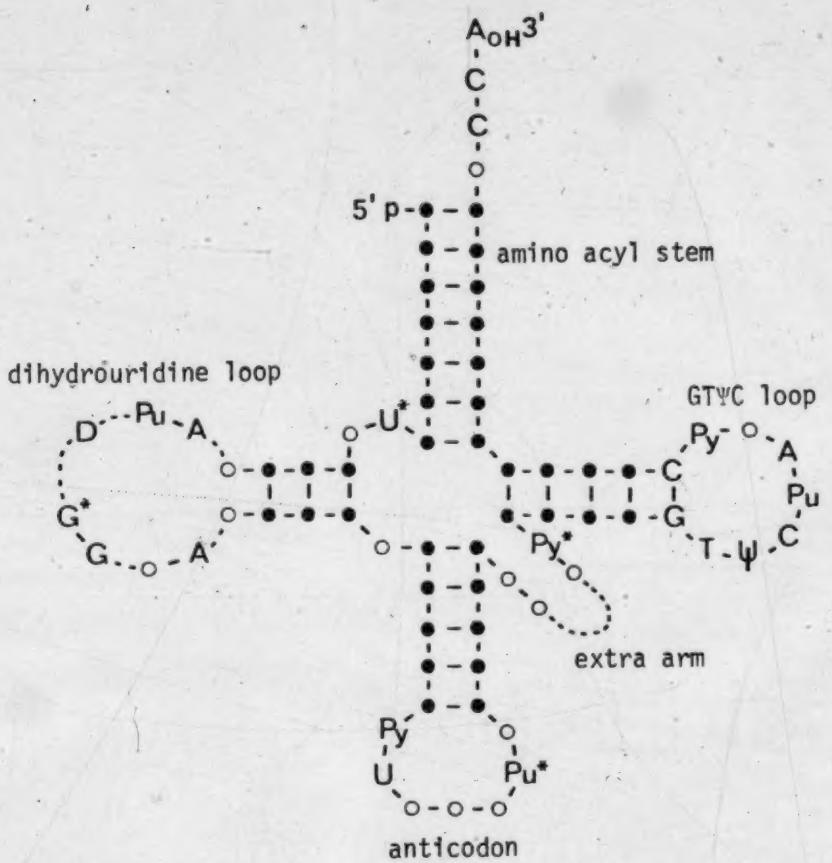

Figure 1

Generalized cloverleaf structure of tRNA

(re-drawn with modifications from Barrell and Clark, 1974)

● - bases in Watson-Crick base pairing

○ - bases not in base pairing in the cloverleaf structure

Pu - purine bases

Py - pyrimidine bases

* - nucleosides often modified

--- - indicates regions with variable lengths

in length (Barrell and Clark, 1974; Clark, 1978). Certain positions of the molecule are also very highly conserved in which only particular nucleosides and/or sequences are present (Barrell and Clark, 1974; Clark, 1978; see Fig. 1). These constant features of the cloverleaf configuration suggest that different parts of the molecule are probably important in particular functions of tRNA.

The biological functions of some of the regions of the tRNA molecule have been quite well studied, e.g. the anti-codon (Crick, 1966) and the 3' aminoacyl terminal (reviewed by Sprinzl and Wagner, 1979 ; Hecht, 1979), but the significance of the other stem and loop regions and the constant features in the functioning of tRNA is still largely unknown. There is evidence which suggests the importance of the sequence of the dihydrouridine stem in tRNA-synthetase recognition (reviewed by Igloi and Cramer, 1978), but a tRNASer lacking the dihydrouridine loop and stem has been identified and can still be aminoacylated (de Bruijn *et al.*, 1981). The GTΨC region has been suggested as a promotor in the transcription of tRNA (K. Roy, personal communication; Koski *et al.*, 1980; Hofstetter *et al.*, 1981), however, direct evidence is still lacking. Recently, the crystalline (three dimensional) structure of several tRNA species has been established (Kim, 1979; Schevitz *et al.* , 1979; Wright *et al.*, 1979; Moras *et al.*, 1980) and these data should help in understanding the structure-function relationships of tRNA (Sundaralingam, 1979).

Another interesting characteristic of tRNA is the presence of a large number of modified nucleosides in the mature molecules (Nishimura, 1978; Nishimura, 1979). To date, over 50 different modified nucleosides have been identified. Some are modified by methylations of the base or the 2'-hydroxyl position of the ribose, while others have very complex modifications (Dunn and Hall, 1975). These modified nucleosides are not randomly distributed in tRNA, but often occupy specific positions in the cloverleaf configuration (see Fig. 1). As an example, the first position of the anticodon as well as the nucleoside 3' to the anticodon are often modified (Nishimura, 1978). It has been suggested that they may be involved directly in base-pairing and/or stabilization of the base-pairing between codon and anticodon (Nishimura, 1978). Certain types of modifications appear only at particular positions, e.g. ribothymidine at the GTΨC-loop and m^2G between the aminoacyl stem and D stem (Gauss and Sprinzl, 1981). The presence of these modified nucleosides at particular positions on tRNA suggests that they probably play an important role in tRNA function, although the data on this are contradictory.

For example, it has been suggested that ribothymidine and pseudouridine could be involved in the binding of tRNA to ribosomes (Sprinzl *et al.*, 1976), but a mutant of *E. coli* lacking ribothymidine grows as well as wild type cells (Svensson *et al.*, 1971). The activities of a number of ribothymidine and/or pseudouridine deficient tRNAs with

respect to aminoacylation and *in vitro* protein synthesis, also do not appear to be impaired (Johnson *et al.*, 1970; Marmor *et al.* 1971; Ofengand *et al.*, 1974). Furthermore, certain species of tRNA from mouse myeloma (Ofengand *et al.*, 1974) and wheat embryo (Marcu *et al.*, 1973) do not have ribothymidine. The presence of N^2-methylguanosine at the 10th nucleoside from the 5' end has been reported to increase the specificity of recognition by aminoacyl-tRNA synthetase (Roe *et al.*, 1973) but evidence is circumstantial.

However, there have been a few cases reported in which a particular nucleoside modification is positively involved in a function of tRNA. The hypermodified nucleoside N^6-(Δ^2-isopentenyl)-2-methylthioadenosine has been suggested as important for the efficient binding of tRNA to ribosomes (Gefter and Russell, 1969). The *trpX* mutant of *E. coli* which is defective in this modification (Vold *et al.*, 1979), was found to decrease the frequency of transcription termination at the attenuator of the tryptophan operon (Yanofsky and Soll, 1977; reviewed by Crawford and Stauffer, 1980). The derepression of the histidine operon in *hisT* mutants of *Salmonella typhimurium* (Chan *et al.*, 1971) in which tRNAHis lacks the Ψ modifications in the anticodon region (Singer *et al.*, 1972) is another of the few examples which demonstrate the involvement of modified nucleosides in tRNA function.

The modified nucleosides are produced post-transcriptionally on tRNA molecules by specific enzymes

(Nishimura, 1978; Nishimura, 1980). It is known that tRNA genes are transcribed into precursor molecules which are subsequently processed to yield the mature molecules (reviewed by Perry, 1976; Daniel *et al.*, 1980; Daniel, 1981). In addition to nucleoside modifications, the maturation process also involves size trimming of these precursors (Altman, 1978). Precursor molecules are larger than the mature tRNAs, carrying extra nucleotides at both the 5' and 3' terminals (Mazzara and McClain, 1980). In some eukaryotic precursor tRNAs, intervening sequences are found adjacent to the anticodon on the 3' side (Goodman *et al.*, 1977; Knapp *et al.*, 1978; O'Farrell *et al.*, 1978; Etcheverry *et al.*, 1979; Olson *et al.*, 1981). These extra nucleotides are removed by the action of specific nucleases (Altman, 1981). For example, RNase P from *E. coli* has been shown to effect the removal of the 5' extra nucleotides (Shimura *et al.*, 1980; Altman *et al.*, 1980). Similarly, 3' processing nucleases have also been identified (Ghosh and Deutscher, 1980). A specific enzyme from yeast has also been implicated in the excision of intervening sequences from eukaryotic tRNA precursors (Ogden *et al.*, 1980; Valenzuela *et al.*, 1980).

Throughout this maturation process, the specific nucleoside modifications are also carried out. A number of these modification enzymes, particularly the tRNA methylases, have been isolated and purified (Nau, 1976), however the details of the whole modification process are

still unknown. Apparently, complete nucleoside modification does not seem to be required for the function of tRNA in protein synthesis (Phillips and Kjellin-Stråby, 1967; Svensson *et al.*, 1971; Singer *et al.*, 1972; Björk and Neidhardt, 1975; Laten *et al.*, 1978). This makes the identification and characterization of mutants in particular modification processes very difficult. Recent data have shown that nucleoside modifications follow a certain order which correlates with size alteration of the precursor molecule (Melton *et al.*, 1980; Nishikura and De Robertis, 1981). These observations could suggest a possible regulatory function of nucleoside modification in the whole maturation process, but no direct evidence is available.

A genetic approach to the study of the functions of tRNA is to analyse the activities of nonsense suppressors. In yeast, as in *E. coli* and other prokaryotes (reviewed by Korner *et al.*, 1978); nonsense suppressors are mutant tRNAs (Hawthorne and Leupold, 1974; Piper *et al.*, 1976; Goodman *et al.*, 1977; Piper, 1980) which have an altered anticodon sequence thus allowing the tRNAs to read one of the termination codons. The occurrence of nonsense codons within a gene results in premature termination during translation and hence a mutant phenotype. Suppressor tRNAs, with the altered coding capacity, can recognize these nonsense codons and allow translation to continue by inserting an amino acid at that position (Capecchi *et al.*, 1975; Gesteland *et al.*, 1976). Therefore, by analysing the efficiencies of a

suppressor on particular nonsense mutations, the activity of that tRNA during translation can be monitored. Of course, other factors which affect the efficiencies of the translation process also have to be considered before any conclusions can be drawn.

If modified nucleosides were involved in the proper functioning of tRNA, then the activity of suppressors might be altered in situations when the full complement of modifications are not present. Indeed, the *mod5-1* mutation of yeast which is defective in isopentenyladenosine modification was isolated by the reduction of the efficiency of a tyrosine inserting nonsense suppressor *SUP7-1* (Laten *et al.*, 1978). Similarly, the *los1-1* mutation, which results in the accumulation of certain precursor tRNAs in yeast, was isolated by screening for the conditional loss of suppression at nonpermissive temperatures (Hopper *et al.*, 1980). This selection scheme was also used in the isolation of a collection of mutations within the *SUP4* tRNATyr gene of *Saccharomyces cerevisiae* which inactivates the suppressor (Kurjan *et al.*, 1980). Genetic studies, accompanied by the appropriate biochemical analyses , are certainly the proper approach to understanding the biosynthesis and activities of tRNA.

To study the biosynthesis and functions of modified nucleosides in tRNA, it will be useful to analyse tRNA molecules lacking the modifications. Mutations that result in the production of undermodified tRNAs are important tools

for both biochemical and genetic analyses. These undermodified tRNAs may be the result of a genetic defect in the modification machinery of the cell and can be identified as isoaccepting tRNA species using high resolution chromatographic techniques. However, isoaccepting tRNAs may also be produced by variation in growth conditions, or due to artefacts of tRNA extraction or chromatographic conditions (Littauer and Iouye, 1973); these do not represent real cellular processes and are not useful for these studies.

Only a few mutants affecting nucleoside modification have been reported. Mutants defective in the production of 5-methyluracil (Björk and Isaksson, 1970; Marinus *et al.*, 1975), 7-methylguanosine (Marinus *et al.*, 1975), 5-methylaminomethyl-2-thiouridine (Marinus *et al.*, 1975), and 4-thiouridine (Ramabhadren *et al.*, 1976) have been isolated in *E. coli* . The *trpX* mutation of *E. coli* , defective in N^6-(Δ^2-isopentenyl)-2-methylthioadenosine modification (Vold *et al.*, 1979) has been implicated in the attenuation of the trp operon (Crawford and Stauffer, 1980). The *hisT* mutation of *Salmonella typhimurium* , defective in Ψ modifications at the anticodon region (Singer *et al.*, 1972), is probably one of the best characterized mutants with respect to its effect on the functioning of tRNA in the regulation of gene expression (Barnes 1978; DiNocera *et al.* , 1978; Johnston *et al.*, 1980). In the yeast, *Saccharomyces cerevisiae* , only two mutations affecting nucleoside

modification have been documented. The *trm1* mutation, defective in N^2-dimethlylguanosine production was identified amongst a collection of methionine auxotrophs (Phillips and Kjellin-Stråby, 1967). The isolation of *mod5-1* has been mentioned in the previous paragraph. Recently, a mutation defective in ribothymidine modification has also been isolated (A. Hopper, personal communication). In the present study, a mutant of *Saccharomyces cerevisiae* defective in the production of dihydrouridine is identified and characterized.

It has been reported that a strain of yeast produces multiple isoaccepting species of tRNATyr as resolved by reversed-phase (RPC-5) chromatography (Bruenn and Jacobson, 1972). The production of isoaccepting tRNAs was also observed for tRNAPhe, tRNAVal and tRNASer and was found to be dependent on growth conditions of the yeast cells (Bell *et al.*, 1978). The first part of this study presents genetic data on the production of these isoaccepting tRNAs and establishes a genetic basis for their production (Bell *et al.*, 1977). The pleiotropic effect of this mutation (designated *mia*) is also examined. Next, the results of physiological studies on the production of the mutant isoacceptors are presented (Lo and Bell, 1981). A precursor product relationship between the mutant isoacceptors and the cognate wild type tRNAs is suggested. The data also indicate *in vivo* activity of the mutant tRNAs in protein synthesis. To continue, by nucleoside composition analyses of some of

the purified isoacceptors, the actual molecular lesion involved in the production of mutant isoacceptors is identified (Lo *et al.*, submitted for publication). Results of *in vitro* poly-U translation assays using the purified tRNAPhe isoacceptors are consistent with the isoacceptors being active during protein synthesis. Finally, the effect of lacking the dihydrouridine modification(s) on the functioning of tRNA is examined. Yeast strains carrying *mia*, the ochre suppressor *SUP4* and a number of ochre mutations were constructed. The efficiency of *SUP4* in the suppression of these ochre mutations is then analysed.

II. Materials & Methods

This chapter is a comprehensive description of all the experimental procedures used. A brief description of some of the procedures will also appear in the pertinent chapters.

Yeast Strains

The genotypes and sources of the parental haploid strains used in this study are listed in Table 1. S288C is a wild type strain which carries a *MIA*$^+$ allele and was used in all cases as the source of wild type tRNAs. XB109-5B was reported to produce multiple isoaccepting tRNA species (Bruenn and Jacobson, 1972; Bell *et al.*, 1978) and was used in the genetic analyses for the segregation of *mia* . Strain D38 carries the mutation *trm1* which results in the accumulation of tRNAs lacking the nucleoside N^2-dimethylguanosine because of an inactive enzyme for this methylation of guanosine (Phillips and Kjellin-Stråby, 1967). Strain J12-9A carries the ochre mutations *ade2-1*, *lys1-1*, *met4-1* and *can1-100* while J15-13C carries *SUP4* in addition to the same ochre mutations in J12-9A (J. Kurjan, personal communication). These two strains are used in the construction of the *mia* (or *MIA*$^+$) *SUP4* strains for the experiments which test the effect of *mia* on the efficiency of *SUP4* .

Table 1. Genotypes of parental haploids

Strain	Genotypes	Source
S288C (wild type)	α *gal2 mal mel*	Dr. K.B. Jacobson
XB109-5B	*a ade5,7-1 lys1-1 leu2-1 met8-1 trp1-1 ura4-1 tyr?-1 ilv1-1 mia*	Dr. J. Bruenn
XB109-5C	*a ade5,7-1 lys1-1 leu2-1 trp1-1 ura4-1 try?-1 ilv1-1 SUP5-1*	Dr. J. Bruenn
XB109-5D	α *ade5,7-1 lys1-1 leu2-1 met8-1 trp1-1 ura4-1 ilv1-1*	Dr. J. Bruenn
D38	*a ade⁻ met⁻ trm1*	Dr. K. Kjellin-Straby
J12-9A	*a ade2-1 lys2-1 trp5-2 can1-100 met4-1 leu1-12 ura1-1*	Dr. J. Kurjan
J15-13C	α *ade2-1 lys2-1 trp5-2 can1-100 met4-1 leu1-12 ura3-1 SUP4*	Dr. J. Kurjan

. The diploid strains constructed for this study are described in Table 2 together with the parents used in each of the crosses. The haploid segregants that were used are listed in Table 3.

Media

All yeast strains were routinely maintained on YEPD which consists of 1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose and 2% bacto-agar. The agar was omitted in liquid medium. The segregation of auxotrophic markers was scored on omission plates which is synthetic complete medium with the specific supplement(s) omitted. Synthetic complete medium consists of 0.67% bacto-yeast nitrogen base (w/o amino acids), 2% dextrose, 2% agar and supplemented as follows (ug/ml final concentration): arginine 20, adenine 20, methionine 20, tryptophan 20, histidine 20, leucine 20, tyrosine 20, phenylalanine 20, uracil 20, isoleucine 20, lysine 30, valine 75, threonine 350 and serine 375 . To score for the segregation of resistance to canavanine, arginine was omitted and replaced by canavanine (40 ug/ml final concentration). Unsupplemented synthetic complete was used as the minimal medium.

Diploids were sporulated on sporulation medium which consists of 0.99% potassium acetate, 0.1% dextrose, 0.25% bacto-yeast extract and 1.5% agar, supplemented with any necessary requirements (same concentration as synthetic complete).

Table 2. Diploid Construction

Diploids	Parents		
JB759	XB109-5B	x	S288C
JB760	XB109-5B	x	S288C
JB836	JB760-1B*	x	JB760-1C*
JB837	JB760-1A*	x	JB760-1D*
RL101	D38	x	JB759-5D
RL113	JB760-1C*	x	J12-9A
RL114	RL113-2*	x	J15-13C

* See Table 3 for genotypes of these strains

Table 3. Genotypes of pertinent haploid segregants used in this study

Strain	Genotypes
JB760-1A	α *lys1-1*
JB760-1B	*a ade5,7-1 leu2-1 met8-1 ura4-1 mia*
JB760-1C	α *tyr7-1 mia*
JB760-1D	*a ade5,7-1 lys1-1 leu2-1 met8-1 ura4-1*
JB760-5A	*a ade5,7-1 lys1-1 met8-1 mia*
JB760-5B	*a leu2-1 tyr7-1 ura4-1 mia*
JB760-5C	α *lys1-1 ura4-1*
JB760-5D	α *ade5,7-1 leu2-1 met8-1 tyr7-1*
JB760-8A	α *leu2-1 met8-1 ura4-1 mia*
JB760-8B	*a ade5,7-1 ura4-1 mia*
JB760-8C	α *lys1-1 tyr7-1 ilv1-1*
JB760-8D	*a ade5,7-1 lys1-1 leu2-1 met8-1 tyr7-1*
JB759-5A	α *ade5,7-1 met8-1 ura4-1*
JB759-5B	*a ade5,7-1 tyr7-1 ura4-1 mia*
JB759-5C	*a lys1-1 leu2-1 met8-1 tyr7-1 ilv1-1*
JB759-5D	α *lys1-1 leu2-1 mia*
JB759-6A	α *lys1-1 leu2-1 met8-1 tyr7-1 ura4-1*
JB759-6B	*a ade5,7-1 lys1-1 leu2-1 mia*
JB759-5C	α *ade5,7-1 met8-1 tyr7-1 ura4-1 mia*
JB759-6D	*a*
JB836-1A	*a ade5,7-1 tyr7-1 mia*

Table 3 (Cont'd)

Strain	Genotype
JB836-1B	*a Leu2-1 met8-1 ura4-1 mia*
JB836-1C	*α ade5,7-1 met8-1 tyr7-1 ura4-1 mia*
JB836-1D	*α leu2-1 mia*
RL101-5B	*α ade⁻ leu2-1 mia trm1*
RL113-2	*a ade2-1 lys2-1 met4-1 can1-100*
RL114-2A	*α ade2-1 lys2-1 met4-1 can1-100 SUP4*
RL114-2B	*α ade2-1 lys2-1 met4-1 can1-100 ura3-1 leu1-12 SUP4 mia*
RL114-3A	*α ade2-1 lys2-1 met4-1 can1-100 ura3-1 SUP4 mia*
RL114-3B	*a ade2-1 lys2-1 met4-1 can1-100 len1-12 SUP4*
RL114-7A	*α ade2-1 lys2-1 met4-1 can1-100 leu1-12 SUP4 mia*
RL114-7C	*a ade2-1 lys2-1 met4-1 can1-100 leu1-12 SUP4*

For the *in vivo* pulse-label and chase experiment, liquid YEPD supplemented with [^{3}H]-uracil (40Ci/mmole, New England Nuclear) at a concentration of 1 uCi/ml was the labelling medium while unsupplemented liquid YEPD was the chase medium.

Genetic Techniques

Diploids were constructed by mixing cells of opposite mating type on a YEPD plate for 4 hr. After observing the appearance of peanut-shaped zygotes under the microscope, the cells were transferred to the proper omission medium to select for diploids in crosses where the parents carry complementing markers. In the construction of auxotrophic homozygotes, selection of diploids by complementation is not possible, and the zygotes were isolated on 3% agar slabs by micromanipulation (Hawthorne and Mortimer, 1960). The putative diploids so obtained were all confirmed by testing for the proper markers and sporulation ability.

To sporulate diploids, cells were transferred onto sporulation medium for 3 days at 30°C, after which tetrads could be observed under the microscope. The tetrads were subjected to glusulase (Endo Laboratories) digestion and then dissected on 3% agar slabs with the aid of micromanipulators (Johnston and Mortimer, 1959). After 2-3 days of growth, the spore clones were replica-plated onto omission media to score for the segregation of auxotrophic markers. Segregation of canavanine resistance was scored on

canavanine plates.

For the isolation of random spores carrying *mia* and the ochre mutations *ade2-1*, *lys2-1*, *met4-1* and *can1-100* , the sporulated diploids were digested for 30 min with glusulase and vortexed vigorously to break up the tetrads. Serial dilutions were done to obtain 100-500 colonies on canavanine plates. Pink colonies (spores carrying *ade2-1*) were picked and then tested for the presence of *lys2-1* and *met4-1*. Finally, the spores were scored for *mia* by extracting the tRNA from individual spore cultures and analyzing them by RPC-5 chromatography (see later sections).

The haploid parent XB109-5B has a clumpy cell phenotype in which aggregates of cells can be observed under the microscope. The test for clumpiness was done simply by examining a fresh culture under the microscope for the presence of cell aggregates.

Yeast Cultures and Growth Kinetics

For the preparation of tRNA, yeast cultures were grown in liquid YEPD at 30°C in a Brunswick shaker-incubator at 200 rpm in aliquots of 250 ml in 2 liter Erlenmeyer flasks. This is the standard shaking condition which favors the appearance of mutant isoaccepting tRNAs in *mia* strains (Bell *et al.*, 1978). All cultures were harvested at late stationary stages (unless otherwise stated) by centrifugation in a Sorvall RC2-B refrigerated centrifuge at 6,000 rpm in a GSA rotor for 10 min.

The growth kinetics of *mia* versus *MIA*$^+$ yeast strains were determined by removing samples (from parallel cultures in liquid YEPD) at timed intervals and measuring the optical density at 550 nm (OD_{550}) after washing the cells once in distilled water. Viable cell counts were also determined from the samples to verify the growth curves obtained by OD_{550}.

To determine the production of mutant isoacceptors in *mia* cultures at different stages of growth, cells were harvested at a specific stage as indicated by an OD_{550} and the tRNA was extracted for chromatography.

Effect of *mia* on Suppression

The efficiency of *SUP4* in the presence of *mia* was examined by comparing the growth kinetics of strains carrying *SUP4 mia* or *SUP4 MIA*$^+$ in liquid omission media which required *SUP4* to suppress particular ochre mutations. Overnight cultures of the proper strains were inoculated into fresh synthetic complete and omission media, and grown at 23°, 30° or 37°C with fast shaking. At timed intervals, samples were removed and the OD_{550} determined. The petri-plate test was done by spotting about 10^4 cells on omission plates and scoring for growth after incubation at the different temperatures.

Preparation of tRNA and Aminoacyl-tRNA Ligases

a) Preparation of tRNA

Crude tRNA from yeast was isolated as in Bell *et al.* (1976) with slight modifications. Only freshly harvested yeast cells were used to ensure minimal degradation of tRNA molecules and the labile modified nucleosides.

Yeast cells were suspended in extraction buffer (1.5 times v/w) together with an equal volume of 88% water-saturated phenol. The extraction buffer was 0.05M Tris-Cl (pH 7.5), 0.05M $MgCl_2$ and 0.01M β-mercaptoethanol. After stirring for 4 hr with a magnetic stirrer at 4°C, the suspension was centrifuged at 10,000 rpm in a SS34 rotor (Sorvall RC2-B centrifuge) for 45 min at 4°C. The upper aqueous layer was carefully removed, mixed with 0.1 volume 4M potassium acetate (pH 4.5), 2.5 volumes cold 95% ethanol and precipitated at -20°C overnight.

The precipitate was collected by centrifugation (10,000 rpm, 10 min, SS34 rotor) and dissolved in a small volume of buffer: 0.05M Tris-Cl (pH 7.5), 0.01M $MgCl_2$ 0.001M EDTA and 0.01M β-mercaptoethenol. This was loaded onto a DEAE-cellulose (Sigma) column pre-equilibrated with the same buffer (size of column used varied depending on the amount of starting material.) About ten column volumes of 0.3M NaCl in buffer were washed through to elute nucleotides and small oligonucleotides as monitored by absorbance at 260 nm (A_{260}). When the A_{260} was less than 0.1, the tRNAs were eluted with 1M NaCl in the same buffer. Fractions with A_{260}

greater than 0.2 were pooled and ethanol precipitated as above.

The tRNAs were recovered by centrifugation, dissolved in a small volume of tRNA storage buffer: 0.05M Tris-Cl (pH 7.5), 0.01M $MgCl_2$, 0.001M EDTA, to give a final concentration of 40-200 A_{260} units/ml and stored at -20°C.

This method of extracting tRNA routinely resulted in a yield of between 12-20 A_{260} units/gm of starting material (20 A_{260} units equal approximately 1 mg tRNA). The preparations were found to contain mainly 4S RNA (greater than 95%) by sucrose gradient and polyacrylamide-urea gel analyses.

b) Preparation of crude aminoacyl-tRNA ligases

Crude aminoacyl-tRNA ligases were prepared as described by Nishimura *et al.* (1967) with modifications. Twenty gm of freshly harvested yeast cells from S288C grown in liquid YEPD until late logarithmic stage were suspended in 20 ml of extraction buffer which consisted of 0.05M Tris-Cl(pH 7.8), 0.01M Mg acetate and 0.01M β-mercaptoethanol. All subsequent steps were carried out at 4°C. The cells were disrupted by 3 passages through a French pressure cell (Aminco) at 17,000 psi and centrifuged at 10,000 rpm (SS34 rotor) for 5 min to remove cell debris. 0.2 volumes of a 5% w/v streptomycin sulfate solution were added dropwise to the extract and stirred for 15 min. The extract was then centrifuged at 30,000 rpm in a Beckman SW 50.1 rotor for 3 hr to sediment

the ribosomes and larger cellular particles. The supernatant was loaded onto a DEAE-cellulose column (1.2 x 10 cm) pre-equilibrated with enzyme buffer (0.05M Tris-Cl pH 7.8, 0.01M Mg acetate, 0.06M KCl and 0.006M β-mercaptoethanol) and washed with 0.15M NaCl in this enzyme buffer. About 50 ml of eluent were collected and brought to 85% saturation with solid ammonium sulfate by stirring. The precipitate was collected by centrifugation at 10,000 rpm (SS34 rotor) for 30 min and dissolved in 10 ml enzyme buffer. It was then dialysed against enzyme buffer overnight, 2 x 2 liters, and finally mixed with an equal volume of cold glycerol. This enzyme preparation could be stored at -20°C for over one year without appreciable loss of the aminoacyl-tRNA ligase activities.

Aminoacylation of tRNAs

The tRNAs were aminoacylated as described in Bruenn and Jacobson (1972) in a modified assay which contained 0.1M Tris-Cl (pH 7.5), 0.02M $MgCl_2$, 6mM ATP (pH 7.0), 0.6mM CTP, 0.1M KCl, 0.01M β-mercaptoethanol, 25 ul yeast aminoacyl-tRNA ligases and 0.5 - 10 A_{260} units of bulk tRNA in a final volume of 100 ul at 37°C for 10 min. The [^{14}C] L- and/or [^{3}H] L- amino acids were present at a final specific activity of 250 and/or 2500 Ci/mole respectively and at concentrations of 20 uM unless otherwise stated. For the aminoacylation of the purified tRNA isoacceptors, < 0.1 A_{260} units of tRNA were used in the assays. Due to their very

rapid aminoacylation, the assays were carried out for only 3 min.

At timed intervals, 10 ul samples were removed, spotted on Whatman 3MM filters and washed in 10% cold trichloroacetic acid (TCA) for 10 min (10 ml/filter). This was followed by 2 washes in 5% cold TCA (10 min each), a wash in cold 95% ethanol (5 min) and a wash in diethyl ether (5 min). The filters were finally air dried for liquid scintillation counting. The acceptance levels of tRNA samples were calculated from the plateau values of the reactions and expressed as pmoles/A_{260} unit.

To prepare aminoacyl-tRNAs for RPC-5 chromatography, the reactions were carried out as above using 0.25 ml yeast aminoacyl-tRNA ligases in a final volume of 1 ml. At the end of the reaction time, the reactions were stopped by adjusting to 0.2M Na acetate (pH 4.6) in a total volume of 5 ml. In the aminoacylation of the purified tRNA isoacceptors, 10 A_{260} units of carrier tRNA were added to increase the recovery of the aminoacyl-tRNAs. This mixture was applied to a DEAE-cellulose column (1.2 x 4 cm) pre-equilibrated with 0.05M Na acetate (pH 4.6), 0.01M $MgCl_2$, 0.001M EDTA and 0.005M β-mercaptoethanol at 4°C. The columns were washed with 50 ml 0.2M NaCl in the same buffer and then the aminoacyl-tRNAs were eluted with 1M NaCl in the same buffer. Fractions of 2 ml each were collected and the radioactivity determined by counting 20 ul samples to locate the aminoacyl-tRNAs. Fractions containing aminoacyl-tRNAs were

pooled and stored at -20°C.

Analytical RPC-5 Chromatography

a) Preparation of resin

RPC-5 resin was prepared as in Pearson, Weiss and Kelmers (1971). Twelve ml of Adogen 464 (Ashland Chemical, Ohio) dissolved in 600 ml chloroform were mixed with 300 gm Plaskon powder (Allied Chemical, N.Y.) in a waring blender. The mixture was homogenized for 5 min using 30 sec bursts to prevent overheating. The homogenate was next poured into a glass tray and left overnight in a fume hood to evaporate the chloroform. Larger crumbs were broken up with a spatula to facilitate complete removal of the chloroform. The resin thus prepared can be stored as a powder for long periods of time.

b) Preparation of columns

Glass columns and accessories were obtained from Chromatronix (California). RPC-5 resin was suspended as a 50% slurry (w/v) in RPC-5 buffer plus 1.5M NaCl. RPC-5 buffer consists of 0.05M Na acetate (pH 4.6), 0.01M $MgCl_2$, 0.001M EDTA and 0.005M β-mercapthethanol. The suspension was mixed at medium speed in a Virtis for a few minutes to break up all the large particles into a very fine slurry. It was then evacuated in a vacuum flask to remove the foam created during the mixing. This slurry was poured into the glass column and packed with 1.5M NaCl in RPC-5 buffer under a

pressure of 250 psi using a reciprocating displacement pump (Milroyal). A packed column of 0.63 X 35 cm has a void volume of approximately 5 ml. After each chromatographic run, the column was regenerated by washing with about 30 ml of 1.5M NaCl in RPC-5 buffer. A column could be used for about 12 chromatograms before resolution was lost.

c) Chromatography

RPC-5 columns in 1.5M NaCl were equilibrated to the NaCl concentration desired for starting the chromatograms for the different aminoacyl-tRNAs by washing with about 30 ml of RPC-5 buffer plus the proper concentration of NaCl. Aminoacyl-tRNA samples (see 'aminoacylation of tRNAs') were also adjusted to the proper NaCl concentration by diluting with RPC-5 buffer before loading onto the columns. The chromatograms were developed with a linear gradient of NaCl in RPC-5 buffer (total volume 400 ml) under a pressure of 250 psi. The gradients used for different aminoacyl-tRNAs will be described with the chromatograms. 60-140 1 ml fractions were collected with an LKB fraction collector and the radioactivity was determined by liquid scintillation counting.

In vivo **Pulse-label of tRNA**

A *mia* strain (JB760-5B) auxotrophic for uracil was used in these experiments. A fresh overnight culture of JB760-5B in liquid YEPD was added to labelling medium at a low

inoculum (approximate 1%). After growing for specific periods (monitored by OD_{550}), the cells were harvested by centrifugation, washed and re-inoculated into the same volume of non-labelling medium for continuous growth until saturation. These cells were then harvested and the tRNA was extracted for chromatography. To determine the distribution of [^{3}H]-label in the different isoaccepting tRNA species during the pulse-label period, tRNA was also extracted from a sample of cells harvested at the end of the pulse period.

In the pulse-label experiments without a chase, the initial inoculation from the overnight culture was into non-labelling medium. After growing for a predetermined time, [^{3}H]-uracil was added (same final concentration as the labelling medium). The cells were harvested after growing for a further timed period and the tRNA was extracted for chromatography.

The [^{3}H]-labelled tRNAs were extracted with phenol and chromatographed on DEAE-cellulose as described before. The crude tRNA samples were then passed through a Benzoylated-DEAE (BD) cellulose column (1.2 x 10 cm) to separate tRNAPhe from bulk tRNA according to the method of Wimmer *et al.* (1968). A detailed description of BD-cellulose chromatography is in the next section. This purification step is necessary to reduce background [^{3}H] counts on subsequent RPC-5 chromatograms used for resolving tRNAPhe isoacceptors aminoacylated with [^{14}C] L-phenylalanine. After BD-cellulose chromatography, the tRNAPhe samples have an

acceptor activity of approximately 600 pmoles per A_{260} unit, which is about a 12-fold purification. The aminoacylation of *in vivo* labelled [^{3}H]-tRNAPhe is as described in the previous section except that [^{14}C] L-phenylalanine was at a concentration of 3.8 uM and a specific activity at 527 Ci/mole. The specific activities of the three tRNAPhe isoacceptors were expressed in terms of total [^{3}H] cpm/[^{14}C] cpm in the area under each of the peaks as resolved in the RPC-5 chromatograms.

Purification of tRNA Isoacceptors

Twenty liters each of JB759-5A *MIA*$^+$ and JB759-5B *mia* were grown up under the standard shaking conditions. About 5000 A_{260} units of crude tRNAs were prepared from each strain by phenol extraction and DEAE-cellulose chromatography as described. Each crude tRNA preparation was deacylated in 100 ml 1M Tris-Cl (pH 8.0) by incubation at 37°C for 2 hr. The tRNAs were recovered by ethanol precipitation.

tRNAPhe and tRNATyr were partially purified from crude tRNA by BD-cellulose chromatography (Wimmer *et al.*, 1968). The partially purified tRNAs were then chromatographed several times on RPC-5 under different chromatographic conditions to remove more contaminants and to separate the different isoacceptors from each other.

a) Purification of tRNAPhe isoacceptors

BD-cellulose was purchased from Regis Biochemical (Illinois) and suspended as a 30-50% slurry (v/w) in 2M NaCl. The slurry was poured into a glass column (2.5 x 25 cm, Chromaflex) and packed with 2M NaCl at a flow rate of 2 ml/min at room temperature using a polystaltic pump (Buchler). Several column volumes of 2M NaCl were washed through the column to settle the cellulose and bring the A_{260} to below 0.1. The column was then equilibrated with 200 ml 0.35M NaCl in BD buffer which is 0.05M Tris-Cl(pH 7.5) and 0.01M $MgCl_2$. The deacylated tRNAs, (5000 A_{260} units) dissolved in 10 ml BD buffer plus 0.35M NaCl, were loaded onto the column and washed with 100 ml of the same buffer and salt molarity. A linear gradient (total volume 1 liter) of NaCl from 0.35M to 1M in BD buffer was then passed through the column to elute the tRNAs. 10 ml fractions were collected and the A_{260} readings determined. After completion of the NaCl gradient, 1M NaCl in BD buffer was passed through the column (fractions collected continuously) to bring the A_{260} below 0.2. A 200 ml linear gradient of 0-20% (v/v) ethanol in 1M NaCl and BD buffer was then applied to the column to elute the remaining tRNAs.

Five ul aliquots of all fractions from the ethanol gradient and every fifth fraction from the NaCl gradient and 1M NaCl wash were assayed for phenylalanine acceptor activity using a modified reaction assay. These aminoacylation assays contained (at a final concentration):

120 mM Tris-Cl (pH 7.5), 22 mM $MgCl_2$, 7.5 mM ATP, 0.75 mM CTP, 120 mM KCl, 12 mM β-mercaptoethanol, 5-10 uM L-phenylalanine (200 Ci/mole and 1000 Ci/mole for [^{14}C] and [^{3}H] L-phenylalanine, respectively), and 15 ul crude yeast aminoacyl-tRNA ligases in a total volume of 50 ul. The reactions were carried out at 37°C for 10 min after which 40 ul were removed, TCA precipitated, washed and dried for liquid scintillation counting as described before. It should be noted that the amount of phenylalanine acceptor activities determined in these assays represent the minimum activities due to the presence of salt and ethanol in the samples which might inhibit the aminoacylation reactions.

Fractions with phenylalanine acceptor activity were pooled, collected by ethanol precipitation and redissolved in tRNA storage buffer. About 250 A_{260} units of tRNA at a phenylalanine acceptor activity of about 600 pmoles/A_{260} were recovered. Fractions from the NaCl gradient with A_{260} above 0.2 were also recovered for the purification of tRNATyr. A BD-cellulose chromatogram for the purification of tRNAPhe is presented in the Appendices.

The partially purified tRNAPhe samples were then fractionated by RPC-5 chromatography in 0.05M Tris-Cl (pH 7.5), 0.01M $MgCl_2$ and 0.001M EDTA using a linear gradient from 0.5M to 1M NaCl. The A_{260} readings and phenylalanine acceptor activities were determined as above. The fractions containing tRNAPhe were pooled and the tRNA recovered by ethanol precipitation. The tRNAPhe samples after this step

have a phenylalanine acceptor activity of about 1200 pmoles/A_{260} and about 120 A_{260} of tRNA were recovered.

To purify the tRNAs further and to separate the three isoacceptors, the tRNAs were aminoacylated with [^{3}H] L-phenylalanine (specific activity 1000 Ci/mole) and chromatographed on RPC-5 using the acetate buffer (pH 4.6) described before. The RPC-5 chromatograms were analyzed by determining the radioactivity in 5 ul aliquots from each fraction and the different isoacceptors were recovered separately. Each tRNAPhe isoacceptor was re-chromatographed separately on RPC-5 again to achieve greater purity from the other isoacceptors. This step was not done for the *MIA*$^+$ tRNAPhe sample since only a single acceptor is present. The purified tRNAs were finally deacylated and stored as described. The final recovered amounts of wild type *MIA*$^+$ and *mia* isoacceptor A, B and C were 31.5, 4.6, 7.8 and 4.8 A_{260} units respectively; all having a phenylalanine acceptor activity above 1200 pmoles/A_{260}. An RPC-5 chromatogram of each of the purified *mia* isoacceptors is presented in Chapter 5.

b) Purification of tRNATyr isoacceptors

The *MIA*$^+$ and *mia* tRNA samples recovered from the NaCl gradient fractions in the BD-cellulose chromatography of tRNAPhe were re-chromatographed on BD-cellulose to purify tRNATyr. About 4500 A_{260} units of each sample were fractionated as described above except that the ethanol

gradient was omitted. Fractions were assayed for tRNATyr acceptor activity with [^{3}H] L-tyrosine using the same modified assay as previously described. About 400 A_{260} units of tRNA with a tyrosine acceptor activity of about 250 pmoles/A_{260} were recovered after this step. The partially purified tRNATyr samples were then chromatographed on RPC-5 in Tris buffer (pH 7.5); about 80 A_{260} units of tRNATyr with a tyrosine acceptor activity of about 1300 pmoles/A_{260} were recovered. These samples were then aminoacylated with [^{3}H] L-tyrosine (1000 Ci/mole) and re-chromatographed on RPC-5 using an acetate buffer (pH 4.6). In the case of the *MIA*$^+$ sample, the single acceptor was recovered after this RPC-5 chromatogram was completed. In the case of the *mia* sample, only the last isoacceptor (called isoacceptor D) was recovered. Absolute purity of this isoacceptor was not required as the contaminating isoacceptors are also mutant (see Chapter 5). The final recovered amounts of *MIA*$^+$ and *mia* isoacceptor D were 12 and 1.2 A_{260} units respectively, with a tyrosine acceptor activity greater than 1400 pmoles/A_{260}. An RPC-5 chromatogram of the purified *mia* isoacceptor D is presented in Chapter 5.

Nucleoside Composition Analyses

a) Digestion of tRNA into nucleosides

The nucleoside compositions of the purified tRNA isoacceptors were analysed by the tritium derivative method as described by Randerath *et al.* (1980). Fifty ug of each

purified tRNA isoacceptor in tRNA storage buffer were ethanol precipitated and re-dissolved in 40 ul distilled water. To this solution was added 0.5 ul 1M $MgCl_2$, 2.5 ul freshly prepared 0.5M bicine-Na (pH 8) and 8 ul of an enzyme solution which contains 10 ug RNase A, 10 ug snake venom phosphodiesterase and 8 ug alkaline phosphatase (all from Sigma). The tRNA was digested at 37°C for 6 hr, distributed in 15 ul aliquots and stored at -60°C.

b) Labelling of nucleosides

Forty ul distilled water and 10 ul 9mM $NaIO_4$ were added to 15 ul of a tRNA digest. This mixture was incubated at 23°C for 2 hr in the dark and cooled on ice for 2 min, after which 1 ul 1M potassium phosphate (pH 6.8) was added and followed immediately by 5 ul [^{3}H] KBH_4. [^{3}H] KBH_4 was obtained from Amersham (3.1 Ci/mmole), dissolved in 0.1M KOH (CO_2-free) at a concentration of 0.1M and stored in aliquots of 10 ul at -60°C. The reactions were incubated in the dark at 23°C for another 2 hr; 100 ul 1N acetic acid were then added to destroy excess borohydride and the solution was evaporated in a stream of filtered air in a fume hood. The dried residue was dissolved in 25 ul 0.1N formic acid and stored at -20°C. The radioactivity of the labelled nucleoside trialcohols was determined by removing 1 ul for liquid scintillation counting and a calculated specific activity of approximately 1.2 Ci/mmole was obtained.

c) Two dimensional chromatography of labelled digest

Five microcuries of a labelled digest (3-4 ul) were applied to the cellulose sheets (Eastman) 2.5 cm from the left-hand and bottom edges. Solvent A was used for chromatography in the first dimension until the solvent front reached the top of the cellulose sheet. Solvent A consists of acetonitrile-4N aqueous ammonia (3.4:1, by volume), freshly prepared. The cellulose sheets were dried in a fume hood, rotated 90° and chromatographed in the second dimension with solvent B, also until the solvent front reached the top of the cellulose sheet. Solvent B consists of t-amyl-alcohol- methyl ethyl ketone-acetonitrile- ethyl acetate-water-formic acid, specific gravity 1.2 (4:2:1.5:2:1.5:0.18, by volume), also freshly made. The sheets were then air dried prior to fluorography.

d) Fluorography and calculation of nucleoside compositions

The air-dried cellulose sheets were coated with a 7% (w/v) solution of 2,5-diphenyloxazole (PPO) in diethyl-ether and marked with [^{14}C]-ink. The fluorograms were exposed at -60° for 2-4 days using Kodak XRP-1 film, after which the films were developed and the spots identified by comparison with a standard nucleoside trialcohol map (Randerath *et al.*, 1980). The nucleoside trialcohol spots were then cut out from the cellulose sheets and quantitated by liquid scintillation counting. The relative amounts of

the nucleosides were calculated according to equation 3 in Randereth *et al.* ,(1980). Three independent determinations were obtained for each tRNA isoacceptor analyzed and the averages are reported in Chapter 5.

Polyacrylamide-urea Gel Electrophoresis

The vertical slab gel electrophoresis apparatus was obtained from Aquaboque Machine and Repair Shop, N.Y. Acrylamide (electrophoresis grade) and N,N'-methylene-bisacrylamide (bisacrylamide) were obtained from Serva and Eastman (Kodak) respectively. 10M urea solutions were purified by passing through a Dowex-50 column to remove contaminating ions. 20% polyacrylamide gels (acrylamide: bisacrylamide 39:1) containing 7M urea were prepared according to Peacock and Dingman (1968) with modifications.

A 30 ml solution was prepared by dissolving 5.85 gm acrylamide and 0.15 gm bisacrylamide in 21 ml 10M urea and 3 ml 10x TEB with stirring and slight warming. The 10x TEB consists of 10.8 gm Tris, 0.926 gm Na_2EDTA and 5.4 gm boric acid in 100 ml distilled water. This solution of monomers was evacuated in a vacuum flask for 10 min. Twelve ul of N,N,N',N', tetramethylethylenediamine (TEMED) and 120 ul of a freshly made 10% ammonium persulfate solution were then added as the catalysts for polymerization. The gel solution was mixed and poured into the gel mold (15 x 12 x 0.15 cm) for polymerization which takes about 15-20 min. The gels were pre-run at 150 volts for 1 hr using 1x TEB as the

running buffer.

The tRNA samples, mixed with a dye solution (50% sucrose, 7M urea, 0.01% xylene cyanol FF), were loaded onto the gels and electrophoresed for 16 hr at 150 volts. The gels were then fixed for 15 min in 1N acetic acid and stained in 0.05% methylene blue in 0.2M Na acetate (pH 4.6) for 20 min. Destaining was achieved in distilled water.

In vitro Poly-U Translation

An S-30 extract was prepared from wheat germ as in Roberts and Paterson (1970) with modifications. Fresh non-toasted wheat germ was purchased from a local health food store and kept in a dessicator at 4°C. Five gm of wheat germ were mixed with 5 gm acid-washed sea sand in 30 ml S-30 buffer made up to 2mM dithiothreitol (DTT) together with 10 ug (a flake) of DNase I (Sigma). S-30 buffer consists of 10mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.8), 60 mM NH_4Cl, 10 mM Mg acetate and 1 mM DTT. This mixture was ground to a fine paste in a pre-chilled mortar and pestle, and centrifuged at 30,000 xg at 4°C for 30 min. The supernatant was passed through a G-25 Sephadex column at 4°C (1.2 x 30 cm) and eluted with S-30 buffer. Turbid fractions were collected, pooled and distributed in 0.5 ml aliquots and stored at -60°C.

The translation assays were also modified from Roberts and Paterson (1970) and contained, at a final concentration, the following ingredients: 20mM HEPES (pH 7.8), 15mM Mg

acetate, 60mM NH_4Cl, 20mM KCl, 2mM DTT, 1mM ATP (pH 7.0), 0.1mM GTP, 10mM creatine phosphate, 20uM [^{3}H] L-phenylalanine (final specific activity 2500 Ci/mole) together with 0.1 ug of a purified tRNA, 4 ug creatine phosphokinase, 20 ug poly-U (Sigma) and 30 ul S-30 extract in a total volume of 100 ul. All ingredients, with the exception of poly-U and [^{3}H] L-phenylalanine, were mixed and pre-incubated for 10 min at 30°C. Addition of poly-U and [^{3}H] L-phenylalanine started the reactions which were carried out for 60 min at 30°C. At timed intervals, 10 ul were removed, spotted on Whatman 3MM filters and immersed immediately in 5% cold TCA (5 ml/filter). After sampling was completed, the flask of filters was transferred to a water bath at 95°C for 10-15 min until the TCA starts to boil. The filters were then washed in cold 5% TCA (same volume) for 15 min, followed by a cold 95% ethanol and an ether wash, and finally air dried for liquid scintillation counting.

III. A Mutant of *Saccharomyces cerevisiae* that Exhibits Multiple Isoacceptors for Several of Its Transfer RNAs

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Summary

A strain of *Saccharomyces cerevisiae*, known to produce multiple isoaccepting forms of several tRNAs which differ from a standard wild type strain, has been studied genetically. The multiple isoaccepting tRNA phenotype behaves as if it is caused by a single recessive mutation. Five tetrads were analyzed and all showed a 2:2 segregation of mutant to wild type profiles for Phe-tRNAPhe. Furthermore, the multiple isoacceptors for the other tRNAs in the mutant strain are probably caused by the same mutation, since Tyr-tRNATyr and Val-tRNAVal also exhibit 2:2 segregation for mutant versus wild type tRNA profiles and the segregation pattern is the same as that for Phe-tRNAPhe.

Introduction

Littauer and Inouye (1973) have reviewed most of the instances where multiple isoaccepting forms of tRNAs have been observed, and they also enumerated many of the known biological roles of tRNA. More recently, the multiple biological roles of tRNA have also been reviewed by Rich and RajBhandary (1976). Although some multiple isoaccepting forms of tRNA are immature or precursor forms caused by a mutation in the organism in question (Waters, Shugart, Yang and Best, 1973), it is also clear that other instances of multiple isoacceptors appear not to have a genetic basis (Wettstein and Stent, 1968; Lazzarini and Santangelo, 1967). It is apparent that the growth conditions used in the various specific systems are very important parameters in producing multiple isoaccepting tRNAs (Littauer and Inouye, 1973; Bell, Jacobson and Shugart, 1978).

It has been reported previously that a strain of *Saccharomyces cerevisiae*, XB109-5B, contains multiple isoaccepting species of tRNATyr as resolved by reversed-phase (RPC-5) chromatography (Bruenn and Jacobson, 1972). In that report the multiple isoacceptors were thought to be formed only in stationary stage cultures and probably represented immature tRNAs. More recently, the observation of multiple isoacceptors of tRNATyr in XB109-5B has been confirmed and multiple isoacceptors were also observed for tRNAPhe, tRNASer, and tRNAVal (Bell, Jacobson and Shugart, 1978). In contrast to the initial observation of multiple

isoacceptors for tRNATyr (Bruenn and Jacobson, 1972), the latter report (Bell, Jacobson and Shugart, 1978) found that, at least for tRNAPhe and tRNATyr, multiple isoacceptors were resolved for tRNAs prepared from logarithmic as well as stationary stage cultures. The appearance of multiple isoacceptors was found to depend on growth conditions in that the more aeration and shaking a culture of XB109-5B was given, the more aberrant the tRNAPhe profiles became. However, if tRNA from a non-shaken culture of XB109-5B was aminoacylated with phenylalanine, tyrosine, serine or valine, a single acceptor was resolved for each type of tRNA, which co-chromatographed with the respective single acceptor resolved for wild type S288C.

In studies that attempt to elucidate possible structure-function relationships between multiple isoaccepting forms of tRNAs and the various biological roles of tRNA, it will be useful to know whether specific multiple isoacceptors have a genetic basis. We wish to report that the observation of multiple isoaccepting forms for several tRNAs in XB109-5B does have a simple genetic basis.

Materials and Methods

Yeast stocks

S288C was obtained from Dr. K.B. Jacobson while XB109-5B, XB109-5C and XB109-5D were kindly provided by Dr. J. Bruenn. The genotypic pedigrees of the three stocks from Dr. Bruenn (i.e. the parental diploid XB109, and its

parents) are given in Bruenn and Jacobson (1972). In our hands the genotype of XB109-5B is: *trp1-1*, *met8-1*, *leu2-1*, *lys1-1*, *ura4-1*, *tyr7-1*, *ade5,7-1*, *ilv1-1*, and mating type *a*, which is consistent with expectations when one looks at the genotype of XB109 (see Bruenn and Jacobson, 1972). XB109-5B tests as *ILV*$^+$ but in tetrads sporulated from diploids of XB109-5B and S288C (which is *ILV*$^+$) we get *ilv*$^-$ segregants. Since XB109 is homozygous for *ilv1-1* (Bruenn and Jacobson, 1972) it is expected that all segregants should be *ilv1-1*. This anomaly is not of importance to the work presented below.

Media and Chemicals

YEPD was used for stock maintenance and routine culturing. It consisted of 1% yeast extract, 2% bacto-peptone, 2% dextrose and 2% agar. The agar is omitted for liquid medium. Synthetic complete (SC) is used as a control for scoring auxotrophic markers and consists of Bacto-yeast nitrogen base (amino acid free) 0.67%, dextrose 2% and agar 2% supplemented as follows: (ug/ml final concentration) arginine 20; lysine 20; adenine 20; methionine 20; tryptophan 20; histidine 20; leucine 30; threonine 350; serine 375; tyrosine 20; phenylalanine 20; valine 75; uracil 20; and isoleucine 20. Omission medium (SC-X) consists of SC with one of the above amino acids omitted and is used to score for segregation of auxotrophic markers. Sporulation medium (SPOR) consists of potassium

acetate, 0.99%; dextrose, 0.1%; yeast extract, 0.25%; and agar, 1.5%.

The Aquasol-2 and the radioactive amino acids were purchased from New England Nuclear.

Genetic Techniques

Diploids were isolated by mixing cells of the opposite mating type for 4 h on YEPD. Zygotes were then isolated on agar slabs by micromanipulation (Hawthorne and Mortimer, 1960). Diploids were transferred to YEPD for one day; the cultures were then replica-plated and grown on SPOR for 3 days (25°C), subjected to snail enzyme digestion and tetrads were dissected with the aid of micromanipulators (Johnston and Mortimer, 1959). Tests for clumpiness were performed simply by examining stationary stage cultures under the microscope for the cell clumping phenotype.

tRNA Preparation and Aminoacylation

Yeast cultures were grown in liquid YEPD at 30°C in a Brunswick shaker-incubator (at 200 rpm) using aliquots of 250 ml in 2 liter Erlenmeyer flasks. These are growth conditions which are known to favour the appearance of multiple isoaccepting tRNAs in XB109-5B. Crude tRNAs and crude aminoacyl-tRNA ligase preparations were obtained as in Bell, Gelugne and Jacobson (1976). The tRNAs were aminoacylated and prepared for reversed-phase chromatography (RPC-5) according to Bruenn and Jacobson (1972). Each of the

[^{3}H] L-amino acids used was present at a final specific activity of 2500 mCi/mmol and an amino acid concentration of 20 uM, while [^{14}C] L-phenylalanine was present at a final specific activity of 250 mCi/mmol and a concentration of 20 uM in aminoacylation reactions. The tRNA concentration in the aminoacylation reactions ranged from 5 to 15 A_{260} nm units/ml reaction volume. At these concentrations, tRNA should be the rate-limiting component of the reaction (Bruenn and Jacobson, 1972) and by time-course measurements we did ensure that a plateau of aminoacylation was achieved for every Phe-tRNAPhe preparation used in these studies.

RPC-5 Chromatography

RPC-5 resin was prepared as in Pearson, Weiss and Kelmers (1971). 140 to 160 1 ml fractions were collected from a column of 0.63 cm x 35 cm with a pressure of 250 lb/in^2 (50 ml/h) using a 400 ml gradient. The gradients for Phe-tRNAPhe and Tyr-tRNATyr were 0.55M NaCl to 1.0M NaCl in the following buffer: 0.05M Na acetate pH 4.6, 0.01M $MgCl_2$, 0.001M EDTA and 0.005M β-mercaptoethanol. The gradients for Asp-tRNAAsp and Val-tRNAVal were from 0.45M NaCl to 0.65M NaCl and otherwise the same as above. The radioactivity of the RPC-5 chromatograms was determined by mixing the 1 ml fractions thoroughly with 9 ml of Aquasol-2 and counting in a Packard model 3003 liquid scintillation counter.

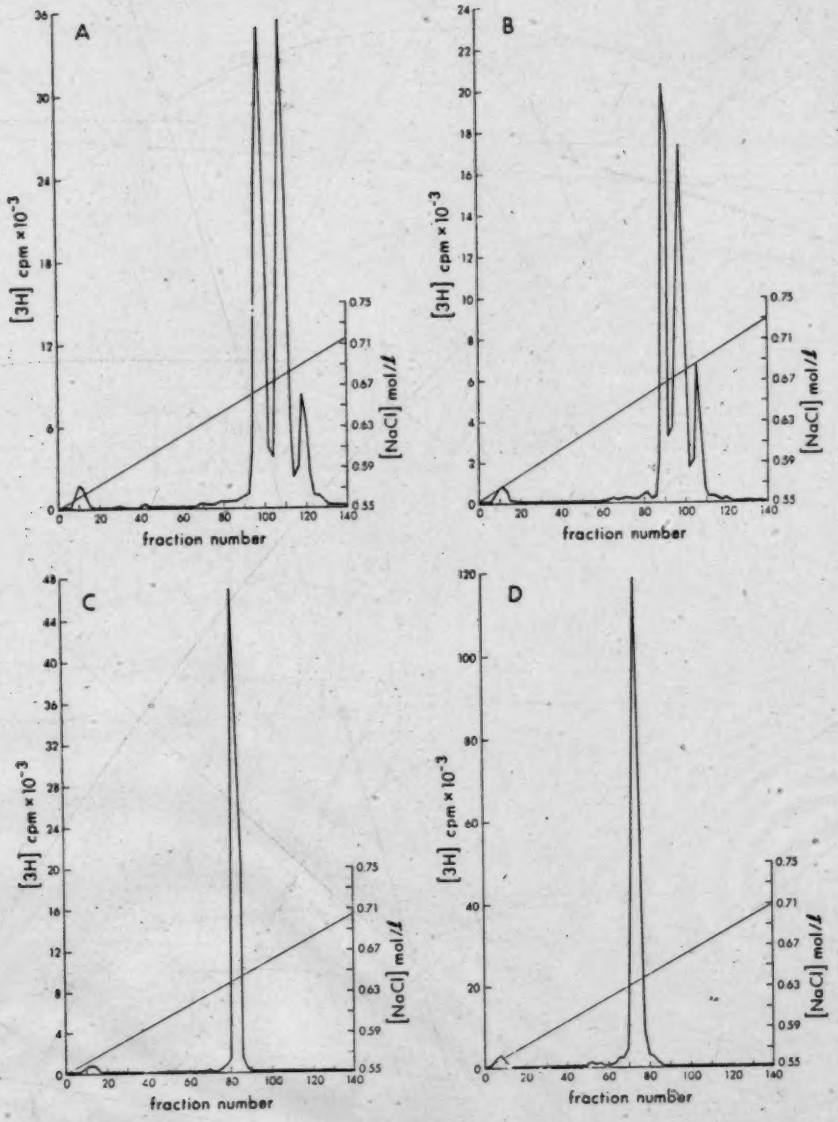
Results

Genetic studies were performed to assess whether the appearance of multiple isoacceptors (MIA) among the tRNAs of XB109-5B had a simple genetic basis. An abstract of some of these experiments has already appeared (Bell, Lo and Quah, 1976). XB109-5B was crossed to S288C (wild type) and several diploids were isolated. Crude tRNA was prepared from two of these diploids (JB759 and JB760), aminoacylated with [^{3}H] L-phenylalanine, and chromatographed on an RPC-5 column. The tRNAPhe profile for each of these diploids was indistinguishable from that of S288C, in that only a single phenylalanine accepting form was resolved. This indicated that the MIA phenotype, if it has a genetic basis, is behaving as if due to a recessive mutation or mutations. The diploids (JB759 and JB760) were then sporulated and several complete tetrads were recovered from each of them.

Five tetrads (two from JB759 and three from JB760) were examined further by growing a culture of each of the twenty spore isolates in liquid medium, extracting crude tRNA from each, aminoacylating each preparation with [^{3}H] L-phenylalanine and chromatographing the product on an RPC-5 column. Each of the five tetrads exhibited a 2:2 segregation pattern for MIA versus a single acceptor for tRNAPhe. Figure 2 illustrates the tRNAPhe profiles obtained from one of the five tetrads analyzed, and these data are identical, in essence, to results obtained for the other four tetrads. A summary diagram of the results for all five tetrads is

Figure 2 A-D

RPC-5 chromatograms portraying the Phe-tRNA^{Phe} profiles of crude tRNA isolated from cultures of one tetrad (JB760-8A, 8B, 8C and 8D). The crude tRNA was isolated, aminoacylated with [^{3}H] L-phenylalanine, and prepared for RPC-5 chromatography as in Methods. The small peak at the beginning of each chromatogram is free phenylalanine derived from deacylation of the tRNA samples between the time of preparation and application to the RPC-5 columns.


A
B
C
D
[3H] cpm × 10⁻³
[NaCl] mol/ℓ
fraction number

presented in Figure 3a. Successive chromatograms from the same RPC-5 column do not always give identical elution positions for similar tRNAs, under the conditions employed in these experiments. This is evident in Figure 2. However, the previous study (Bell, Jacobson and Shugart, 1978) indicated that whenever MIA tRNAPhe was co-chromatographed with differentially labelled single acceptor tRNAPhe from S288C, the first of the multiple isoacceptors to be eluted for mutant tRNAPhe eluted in the same fractions as the single form from wild type. Furthermore, in the present study we find that the first of the multiple isoacceptors to be eluted from tRNA of the MIA spores co-chromatographs with the single acceptor resolved in the non-MIA sister spores (Fig. 4). Finally, we also know that the single tRNAPhe acceptor resolved from S288C tRNA co-chromatographs with the single acceptor for tRNAPhe which is produced in half the segregants from the five tetrads studied (i.e. JB759-5A), results not shown.

XB109-5B is auxotrophic for several nutritional requirements (Materials and Methods). This auxotrophy is due to a series of suppressible ochre and amber mutations, but XB109-5B is ochre and amber suppressor free (Bruenn and Jacobson, 1972). Our results confirm that the ochre and amber mutations are present and expressed. Therefore, we can exclude the presence of ochre or amber suppressors as the explanation for the observed extra isoacceptors. The presence of several tRNAs exhibiting the multiple

Figure 3a and b

Summary diagram of the $tRNA^{Phe}$ phenotypes (as resolved by RPC-5 chromatography) of all crosses and their results (from Tables 4 and 5). All peaks are representative of actual results (e.g. Fig. 2) and were produced by aminoacylating crude tRNAs with [^{3}H]L-phenylalanine and chromatographing the product. a) a representative of the five 2:2 tetrads. b) represents the one 4:0 tetrad.

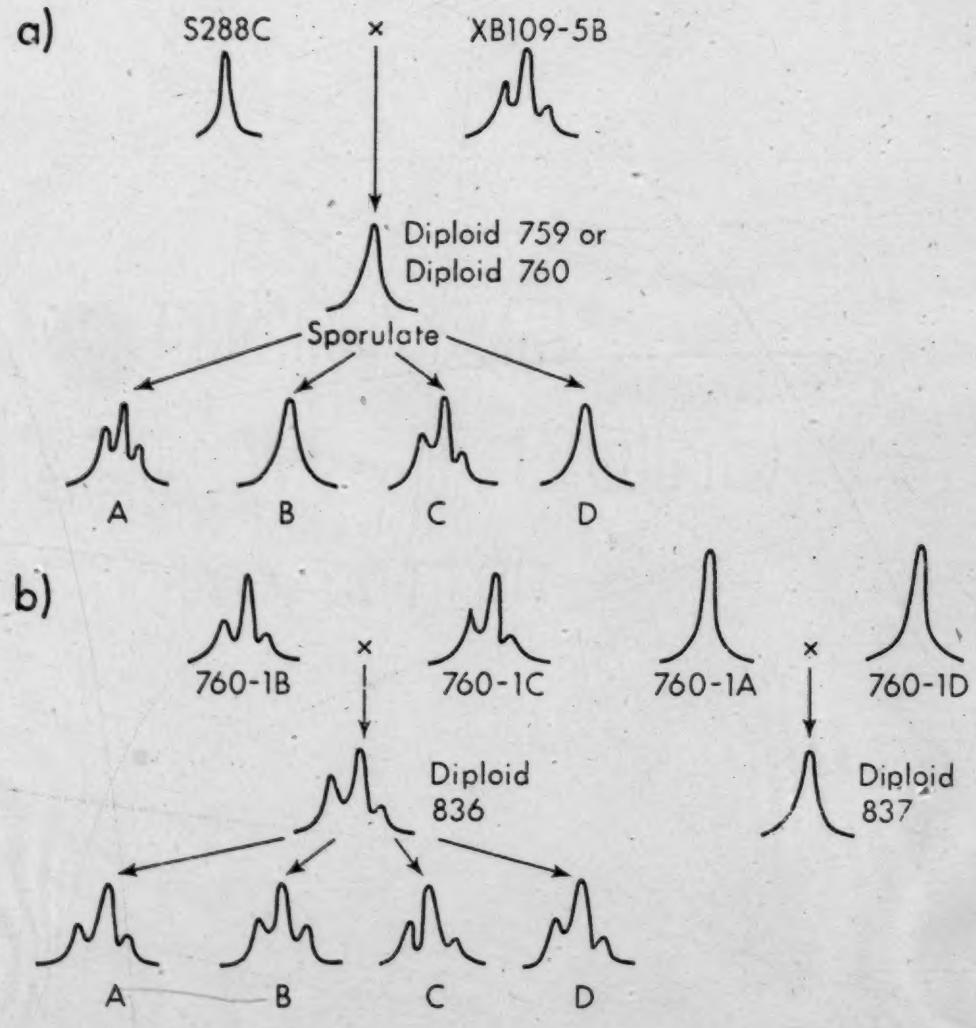
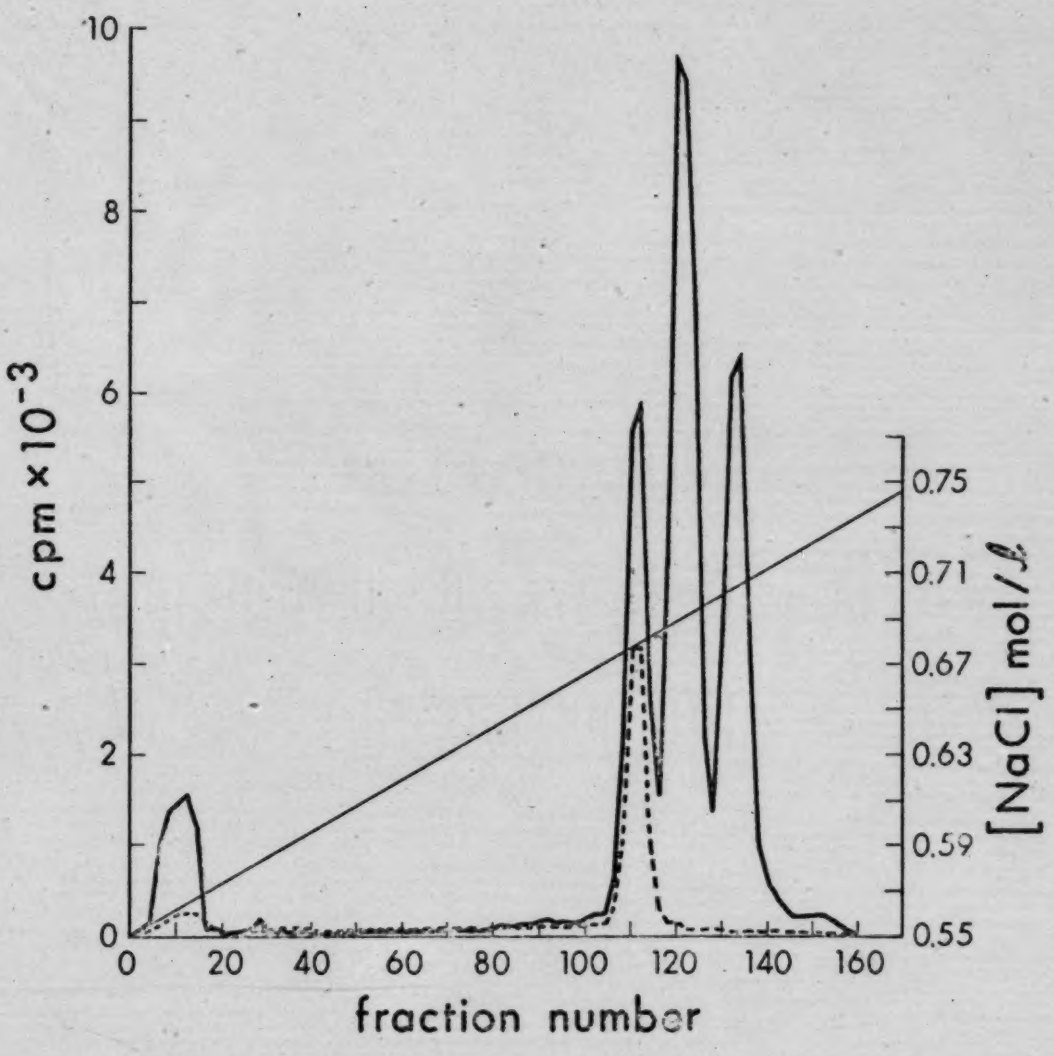

a)
S288C
×
XB109-5B
Diploid 759 or
Diploid 760
Sporulate
A
B
C
D
b)
760-1B
×
760-1C
760-1A
×
760-1D
Diploid
836
Diploid
837
A
B
C
D

Figure 4

RPC-5 co-chromatogram portraying the Phe-$tRNA^{Phe}$ profiles of crude tRNA isolated from sister spores of a tetrad which is segregating for MIA and wild type tRNA. (———) represents [^{3}H] L-phenylalanyl-$tRNA^{Phe}$ from JB759-5B, and (----) represents [^{14}C] L-phenylalanyl-$tRNA^{Phe}$ from JB759-5A. The crude tRNA was isolated and treated as in Figure 2.


10
8
6
4
2
0
cpm x 10⁻³
0.75
0.71
0.67
0.63
0.59
0.55
[NaCl] mol/ℓ
0
20
40
60
80
100
120
140
160
fraction number

isoacceptors is also inconsistent with an informational suppressor being the explanation. Furthermore, two sister spores of XB109-5B have been analysed. XB109-5C contains the amber suppressor *SUP5-1* and XB109-5D does not. A single acceptor for tRNAPhe was resolved on RPC-5 columns for both of these strains (this study; and Bell, Jacobson and Shugart, 1978).

Table 4 presents the complete segregation data for the five tetrads studied. The nutritional requirements segregated 2:2 with the exception of the *ilv* marker. It is likely that a suppressor specific for *ilv* is segregating in these tetrads, as such suppressors are known to exist (Kakar, 1963). Table 4 also shows that the MIA tRNAPhe is segregating independently of all other markers.

Mating-type also segregated from MIA thus making possible a mating between two MIA haploids. JB760-1B was crossed to JB760-1C and JB760-1A was crossed to JB760-1D. The diploid produced from the first mating (JB836) exhibited MIA tRNAPhe while the diploid produced from the latter mating (JB837) produced the expected wild type single acceptor for tRNAPhe. JB836 was sporulated and one complete tetrad was examined. All four spores (JB836-1A, 1B, 1C, 1D) exhibited MIA tRNAPhe (Fig. 3b). Table 5 presents the segregation pattern for the other markers of this tetrad. All markers showed a 2:2 segregation, demonstrating that we are studying a true tetrad. Because the parents of JB836 (i.e. JB760-1B and JB760-1C) are both *LYS*$^+$, the data in

Table 4. Segregation data for auxotrophic markers and tRNA isoacceptor phenotypes among tetrads from sporulated diploids that arose from matings of XB109-5B with S288C

	ade	lys	leu	met	tyr	ura	ilv	MC	YEPG	Mating type	'clumpy'	$tRNA^{Phe}$	$tRNA^{Tyr}$	$tRNA^{Val}$	$tRNA^{Asp}$
760-1A	+[a]	-	+	+	+	+	+	+	+	α	+[b]	S[c]	/[d]	/	/
1B	-	+	-	-	+	-	+	+	+	a	+	M	/	/	/
1C	+	+	+	+	-	+	+	+	+	α	-	M	/	/	/
1D	-	-	-	-	-	-	+	+	+	a	-	S	/	/	/
760-5A	-	-	+	-	+	+	-	+	+	a	-	M	/	/	/
5B	+	+	-	+	-	-	+	+	+	a	-	M	/	/	/
5C	+	-	+	+	+	-	+	+	+	α	+	S	/	/	/
5D	-	+	-	-	-	+	+	+	+	α	+	S	/	/	/
760-8A	+	+	-	-	+	-	+	+	+	α	-	M	M	M	S
8B	-	+	+	+	+	-	+	+	+	a	+	M	M	M	S
8C	+	-	+	+	-	+	-	+	+	α	-	S	S	S	S
8D	-	-	-	-	-	+	+	+	+	a	+	S	S	S	S
759-5A	-	+	+	-	+	-	+	+	+	α	-	S	/	/	/
5B	-	+	+	+	-	+	+	+	+	a	-	M	/	/	/
5C	+	-	-	-	-	-	-	+	+	a	+	S	/	/	/
5D	+	-	-	+	+	+	+	+	+	α	+	M	/	/	/

Table 4 (Cont'd)

	ade	lys	leu	met	tyr	ura	ilv	MC	YEPG	Mating type	'clumpy'	$tRNA^{Phe}$	$tRNA^{Tyr}$	$tRNA^{Val}$	$tRNA^{Asp}$
759-6A	+	-	-	-	-	-	+	+	+	α	-	S	/	/	/
6B	-	-	-	+	+	+	-	+	+	α	+	M	/	/	/
6C	-	+	+	-	-	-	+	+	+	α	+	M	/	/	/
6D	+	+	+	+	+	+	+	+	+	α	-	S	/	/	/

a For the auxotrophic markers a plus (+) refers to a prototrophic segregant while a minus (-) refers to an auxotrophic segregant

b For the 'clumpy' phenotype a plus (+) is a segregant exhibiting the 'clumpy' phenotype while a minus (-) represents a nonclumping segregant

c For the tRNA phenotype, an S refers to a segregant with a single acceptor resolved for that type of tRNA while an M refers to a segregant exhibiting the multiple isoacceptor phenotype

d An oblique stroke refers to those cases for which no data were collected

Table 5. Segregation data of auxotrophic markers and the $tRNA^{Phe}$ phenotype from one tetrad of JB836[a]

	ade	lys	leu	met	typ	ura	$tRNA^{Phe}$
836-1A	-[b]	+	+	+	-	+	M[b]
836-1B	+	+	-	-	-	-	M
836-1C	-	+	+	-	+	-	M
836-1D	+	+	-	+	+	+	M

[a] The parents of JB836 are JB760-1B and JB760-1C and their genotypes are given in Table 4.

[b] The symbols are as in Table 4.

Table 5 are expected.

Initially, multiple isoacceptors for XB109-5B tRNAs were observed for four of the five kinds of tRNA studied. Tranafer RNATyr, tRNAPhe, tRNAVal, and tRNASer each exhibited multiple isoaccepting species on RPC-5 columns while S288C exhibited one form for each of these tRNAs. Transfer RNAAsp exhibited only one form in the mutant as well as the wild type strain (Bell, Jacobson and Shugart, 1978). One of the five tetrads that gave 2:2 segregation for MIA versus a single acceptor for tRNAPhe (JB760-8A, B, C and D) was examined further to see whether the segregation patterns for these other tRNAs correlated with that for tRNAPhe. The tRNASer profile was not examined but tRNATyr and tRNAVal each segregated 2:2 for MIA versus a single acceptor and the pattern was the same as that for tRNAPhe. As expected, tRNAAsp exhibited a single acceptor in each of the four spore cultures since MIA had not previously been observed for this tRNA (Bell, Jacobson and Shugart, 1978). These results are included in Table 4.

XB109-5B has a clumpy phenotype which segregated 2:2 in our crosses (see Table 4; and J. Lemontt, personal communication). This phenotype segregated independently from MIA.

Therefore, none of the markers that XB109-5B is known to contain has proven useful in locating the genetic locus or loci controlling MIA or as a 'tag' in following its segregation.

Discussion

Diploids produced from mating XB109-5B to S288C exhibit a tRNAPhe profile like that of the wild type S288C parent, suggesting that the mutant phenotype (MIA) of XB109-5B is recessive. Results obtained from tetrad analyses of these diploids showed a 2:2 segregation for MIA versus a wild type tRNA pattern. Moreover, a diploid made from two MIA haploid strains is also mutant in phenotype as are its four meiotic products. This result is consistent with MIA having a simple and stable genetic basis. Since we have analysed only five tetrads we cannot distinguish whether MIA tRNA is determined by a single gene or by two closely linked genes (less than ten map units apart). The present lack of a marker tightly linked to the gene(s) causing MIA tRNA (Table 4) makes it laborious and costly to expand our data. Therefore, at this time, mapping of the gene(s) for MIA is not feasible.

The fact that MIA does segregate in crosses allows us to exclude most trivial causes, such as artefacts in the preparation of the tRNA itself, as bases for explaining the phenomenon. Previous data were insufficient to conclude that MIA tRNA has a genetic basis (Bell, Jacobson and Shugart, 1978). However, it was known that the production of multiple isoacceptors was strain specific and appeared to be a stable phenotype; therefore, it seemed highly likely that a genetic basis might be found. We propose the three letter abbreviation *mia* for the genotypic symbol of the gene causing the MIA phenotype, in accordance with the recently

published yeast mutant directory (Plischke, von Borstel, Mortimer and Cohn, 1976), on the assumption that a single locus probably determines the character.

Since the first isoacceptor eluted from XB109-5B tRNA (for any of the tRNAs studied that gave multiple isoacceptors) always co-chromatographed with the cognate single acceptor in S288C (Bell, Jacobson and Shugart, 1978), it is tempting to speculate that the mutational lesion is of the 'leaky' missense variety; that is to say that some wild type product is always produced. The implication is that the lesion results in undermodified tRNAs due to a mutation in a gene specifying some modification enzyme. However, two lines of evidence from the previous paper mitigate against this conclusion.

One reason is that you might expect very late stationary cultures to begin to convert the mutant tRNAs to the wild type form since the cultures are no longer growing rapidly and a mutant, but still functional, enzyme could begin to catch-up in performing its particular function. This does not appear to be the case since stationary stage cultures still exhibit MIA tRNA. Further physiological and biochemical studies are in progress to see if the multiple isoacceptors are truly under-modified tRNA molecules or whether they may represent biological 'dead-end' products which may still be aminoacylated but not converted to normal tRNAs. If the latter alternative is assumed to be correct then the continued appearance of the MIA tRNA in late

stationary cultures is not inconsistent with the genetic lesion being of the 'leaky' missense variety. Then, the explanation could be that the lesion results in the accumulation of an immature form which is then aberrantly modified to some 'dead-end' forms which can never be converted to wild type tRNA, even if a partially functional enzyme is present to 'catch-up' at the late stationary stage. It should also prove informative to ask whether the extra acceptors are still functional in protein synthesis.

The second reason is that the nucleoside digestion data in Bell, Jacobson and Shugart (1978) suggest that the XB109-5B tRNAPhe isoacceptor that co-chromatographs with tRNAPhe from S288C may not be wild type. This is because the technique used gave the expected molar composition of nucleosides for purchased purified wild type tRNAPhe but did not give the expected molar composition for any of the isoacceptors in XB109-5B. This result should be interpreted with caution, however, since that study did not include a nucleoside digest for S288C tRNAPhe and it could be the tRNA purification technique that led to the equivocal results.

Further genetic experiments which are in progress could also shed some light on this problem. We are currently attempting to ascertain whether the MIA phenotype is suppressible, which may indirectly tell us whether it is due to a missense mutation.

There is a paucity of information on mutants affecting tRNA in *Saccharomyces cerevisiae*. However, Phillips and

Kjellin-Stråby (1967) and Kjellin-Stråby and Phillips (1968) have reported a strain of yeast with methyl deficient tRNA and this strain lacks an enzyme that methylates guanosine to form N^2-dimethylguanosine. We do not think that XB109-5B is mutant in the same function, since we find that the segregation pattern of MIA tRNAVal correlates with tRNAPhe and tRNATyr suggesting that the cause of the extra isoacceptors is probably the same for all three tRNAs. Transfer RNAVal lacks N^2-dimethylguanosine in its primary sequence while tRNAPhe, tRNATyr and tRNASer contain this nucleoside (Barell and Clark, 1974). Both the previous study (Bell, Jacobson and Shugart, 1978) and this study find that XB109-5B produces a single acceptor for tRNAAsp. Considering the primary sequences (Barrell and Clark, 1974) for all the tRNAs analysed in the present studies, it is not possible at this time to postulate a specific single mutational lesion to account for the patterns observed, and yet the genetic data suggest that there probably is one. At this point we feel confident that MIA has a genetic basis but we do not know whether the mutant is a lesion in tRNA biosynthesis *per se*, or whether we are studying a secondary effect - somewhat analogous to the production of undermodified tRNA in a *rel*- strain of *E. coli* (Waters, Shugart, Yang, and Best, 1973).

As well as attempting to answer whether the MIA phenotype is suppressible, further studies are also aimed at answering whether this particular mutant that affects tRNA might not also affect tRNA mediated informational

suppression. It is now firmly established in yeast, as it was previously for procaryotes, that altered tRNAs are the mediators of informational suppression (Capecchi, Hughes and Wahl, 1975; Gesteland, Wolfner, Grisafi, Fink, Botstein and Roth, 1976). Therefore, it is reasonable to expect that any altered tRNA is a potential candidate to affect suppression either positively or negatively. Antisuppressors are known and reasonably well characterized genetically in *Saccharomyces cerevisiae* (McCready and Cox, 1973). However, as these authors suggest, the molecular bases of antisuppressors, even though likely to be heterogeneous, are not specifically known. By studying mutants such as XB109-5B it is quite possible that one may gain some insight into at least one molecular basis for antisuppressors.

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IV. Characterization of a Mutation in *Saccharomyces cerevisiae* that Produces Mutant Isoaccepting tRNAs for Several of its tRNA Species: Physiological Studies

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Summary

Genetic data presented in Bell *et al.* (1977) demonstrated that a mutation in *Saccharomyces cerevisiae* (designated *mia*) is responsible for the production of mutant isoaccepting tRNA molecules for some tRNA species. Besides extending this phenotype to other tRNAs, we have shown that mutant isoacceptors are produced at the expense of the normal levels of wild type tRNA and that the presence of mutant isoacceptors has no adverse effects on strains harbouring the mutation. The observation that *mia* strains have mutant isoacceptors as the predominant tRNA species under certain growth conditions suggests that mutant isoacceptors are biologically active molecules. Pulse-label and chase experiments indicate that mutant isoacceptors are slowly converted to wild type tRNA molecules *in vivo* , suggesting that they could be precursor molecules. This is

consistent with the hypothesis that *mia* is defective in a modification process in the maturation of tRNA molecules. Analysis of a double mutant that produces *mia* isoacceptors which also lack N^2-dimethylguanine shows that some of the modifications to tRNA moleucles need not follow a specific sequence.

Introduction

One of the most interesting characteristics of transfer RNA is the presence of many modified bases on the molecule. These unusual bases are produced on the primary transcripts by modification enzymes (Söll, 1971; Altman, 1975; Nau, 1976) and often occupy specific positions on the tRNA molecules (Dunn and Hall, 1975; Sodd, 1975; Nishimura, 1978). Some of the enzymes involved in the modification process have been identified and characterized (Cortese *et al.*, 1974), but the sequence of the steps in the modification process is still largely unknown (Davis and Nierlich, 1974), although evidence is accumulating (Melton *et al.*, 1980). The major function of tRNA is in protein synthesis, but the absence of some of the modified bases on the tRNA molecule does not seem to impair this function (Singer *et al.*, 1972). There have been speculations about the functions of modified bases on the tRNA molecules, but the information is still incomplete (LaRossa and Söll, 1978).

To study the functions of modified bases on tRNA, it is useful to obtain mutants defective in tRNA modifications. All mutations that result in the production of isoaccepting tRNA species are potential tools for these studies since unusual acceptors may represent under-modified tRNA molecules. It has been reported by Bruenn and Jacobson (1972) that a strain of *Saccharomyces cerevisiae* , XB109-5B, produces multiple isoaccepting species of tRNATyr as resolved by reversed-phase (RPC-5) chromatography. Subsequently, Bell *et al.* (1978) also observed multiple isoacceptors for tRNAPhe, tRNASer and tRNAVal. The production of multiple isoacceptors was found to be dependent on the growth conditions of the yeast cultures. Genetic analyses by Bell *et al.* (1977) established firmly that the production of the extra isoacceptors in XB109-5B has a genetic basis. The mutation was designated *mia*. It was observed, in all cases where multiple isoacceptors were resolved, that the wild type acceptor was also present. *Mia* was postulated to be a leaky mutation in a gene specifying some modification enzyme.

In this report we have further characterized *mia* and the mutant isoacceptors. Our data show that mutant isoacceptors are produced at the expense of the normal levels of wild type tRNA and that they are biologically active molecules. The results also show that mutant isoacceptors can be converted to wild type tRNA, and are consistent with the hypothesis that isoacceptors are

undermodified, or aberrantly modified, tRNAs accumulated due to a defect in a modification process.

Experimental Procedures

Yeast Strains, Culture Media and Genetic Techniques

All strains have been described in Bell *et al.* (1977) with the exception of strain D38 which was obtained from Dr. K. Kjellin-Stråby. Strain D38 carries the *trm1* mutation which results in the accumulation of tRNAs lacking the base N^2-dimethylguanine because of an inactive enzyme for this methylation of guanine (Phillips and Kjellin-Stråby, 1967).

All culture media used for the routine growth or segregation tests of auxotrophic markers, as well as genetic techniques, are as described in Bell *et al.* (1977).

Growth Conditions

The standard growth conditions which favour the production of tRNA mutant isoacceptors in *mia* strains are described in Bell *et al.* (1977), and were used in the growing of all yeast cultures herein unless otherwise stated.

The growth kinetics of *mia* versus *MIA*$^+$ yeast strains were determined by removing samples (from parallel cultures in liquid YEPD) at timed intervals and measuring the optical density at 550 nm (OD_{550}) after washing the cells once in distilled water. Viable cell counts were also determined from the samples to verify the growth curves obtained by

OD_{550} readings.

To determine the production of mutant isoaceptors in *mia* cultures at different stages of growth, cells were harvested at a specific stage as indicated by an OD_{550}, and the tRNA was extracted for chromatography.

In Vivo Pulse-label and Chase of tRNA in *mia*

A *mia* strain (JB760-5B) auxotrophic for uracil was used in these experiments. Liquid YEPD supplemented with [^{3}H]-uracil (40 Ci/mmole, New England Nuclear) at a concentration of 1 uCi/ml was the labelling medium while unsupplemented YEPD was the non-labelling medium.

A fresh overnight culture of JB760-5B in liquid YEPD was added to labelling medium at a low inoculum (<1%). After growing for specific periods (monitored by OD_{550}) , the cells were harvested by centrifugation, washed and re-inoculated into the same volume of non-labelling medium for continuous growth until saturation was achieved. These cells were then harvested and the tRNA was extracted for chromatography. To determine the distribution of [^{3}H]-label in the different isoaccepting tRNA species during the pulse-label period, tRNA was also extracted from a sample of cells harvested at the end of the pulse period.

In the pulse-label experiments without a chase, the initial inoculum from the overnight culture was into nonlabelling medium. After growing for a certain period, [^{3}H]-uracil was added (same final concentration as above).

The cells were harvested after growing for another timed period and the tRNA was extracted for chromatography.

Extraction and Chromatography of tRNA

Crude tRNAs were extracted from yeast cells and aminoacylated with radioactive amino acids as in Bell *et al.* (1977). RPC-5 columns and chromatograms were prepared as in Bell *et al.* (1977). The NaCl gradients for Met-tRNAMet, His-tRNAHis, Gly-tRNAGly, and Leu-tRNALeu were 0.5 - 0.9M, 0.55 - 0.8M, 0.5 - 0.7M, and 0.5 - 1.0M, respectively.

The crude tRNA samples which were labelled with [^{3}H]-uracil were passed through a BD-cellulose column to separate tRNAPhe from bulk tRNA, according to the method of Wimmer *et al.* (1968). This purification step is necessary to reduce background [^{3}H] counts on RPC-5 chromatograms used for resolving tRNAPhe when it is aminoacylated with [^{14}C] L-phenylalanine. After BD-cellulose chromatography, the tRNAPhe samples have an acceptance of 600 pmoles of phenylalanine per A_{260} nm unit, which is about a 12-fold purification. The specific activities of the three isoacceptors of tRNAPhe were expressed in terms of total [^{3}H] cpm / [^{14}C] cpm in the area under each of the peaks as resolved in the RPC-5 chromatograms.

Methylation of Crude tRNAs

Crude yeast methylases were prepared from strain S288C as in Björk and Svennson (1969). If not used immediately,

the extracts were mixed with an equal volume of cold glycerol and stored at -20°C. Under these conditions the extracts were stable for several months. The assay conditions were also as in Björk and Svennson (1969) with the following modifications. Thirty ul of crude yeast methylase extract and 0 - 3.5 A_{260} nm units of yeast tRNA were incubated in a total volume of 210 ul containing 10 uM S-[methyl-^{14}C]adenosylmethionine at 50 mCi/mmole (New England Nuclear). The reaction was carried out for 120 min at 35°C.

Results

Pleiotropic Effects of *mia*

Studies of the *mia* phenotype (Bell *et al.*, 1977) have been extended to several other tRNA species from XB109-5B by comparisons with the respective wild type tRNA phenotypes of S288C. The segregation of the tRNA phenotype for these additional tRNAs was also analysed. The results are summarized in Table 6, while RPC-5 chromatograms of *mia* tRNAs are displayed in Figure 5 for those tRNAs not previously presented (Bell *et al.*, 1977; Bell *et al.*, 1978). Strains containing *mia* exhibit an extra acceptor for tRNAMet and tRNALeu on RPC-5 chromatograms, while the tRNAGly and tRNAHis profiles in *mia* strains are indistinguishable from those of *MIA*$^+$ strains.

Multiple acceptors are normal for some tRNAs in S288C but, in all cases, tRNAs affected by *mia* exhibit extra

Table 6. Diagrammatical summary of the segregation analyses of tRNA isoacceptor phenotypes in a tetrad produced from a *MIA*$^+$ strain (S288C) and a *mia* strain (XB109-5B)

a, b Among the tRNA species examined, those affected by *mia* are indicated by a, and those not affected by *mia* are indicated by b.

c All diagrams are representative of actual profiles (see Figure 5, Bell *et al.*, 1977, and Bell *et al.*, 1978).

d The data allow one to score the tetrad as follows: JB760-8A and JB760-8B are *mia*, while JB760-8C and JB760-8D are *MIA*$^+$. The segregation of auxotrophic markers in this tetrad is presented in Bell *et al.* (1977) and in Table 4.

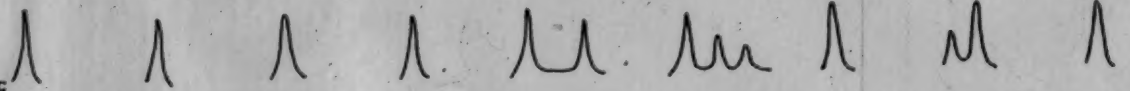
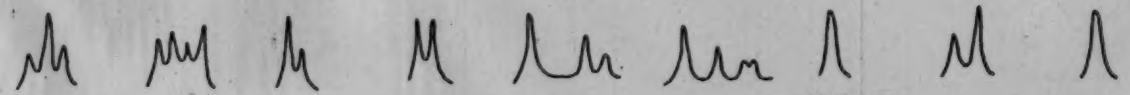
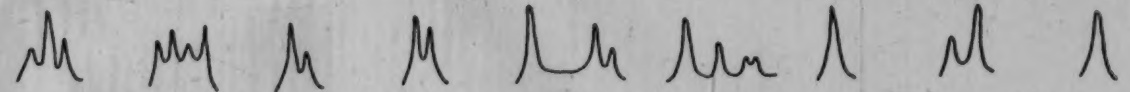
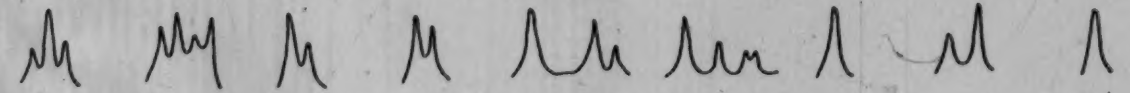
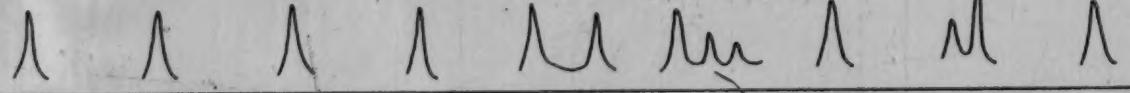
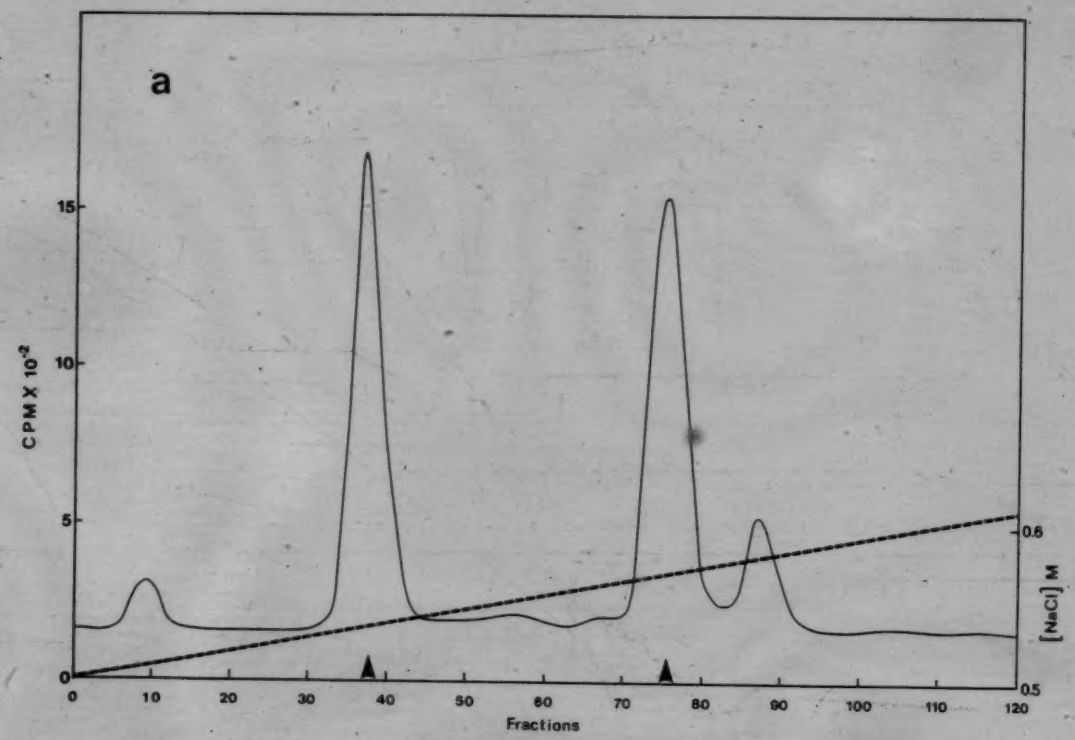
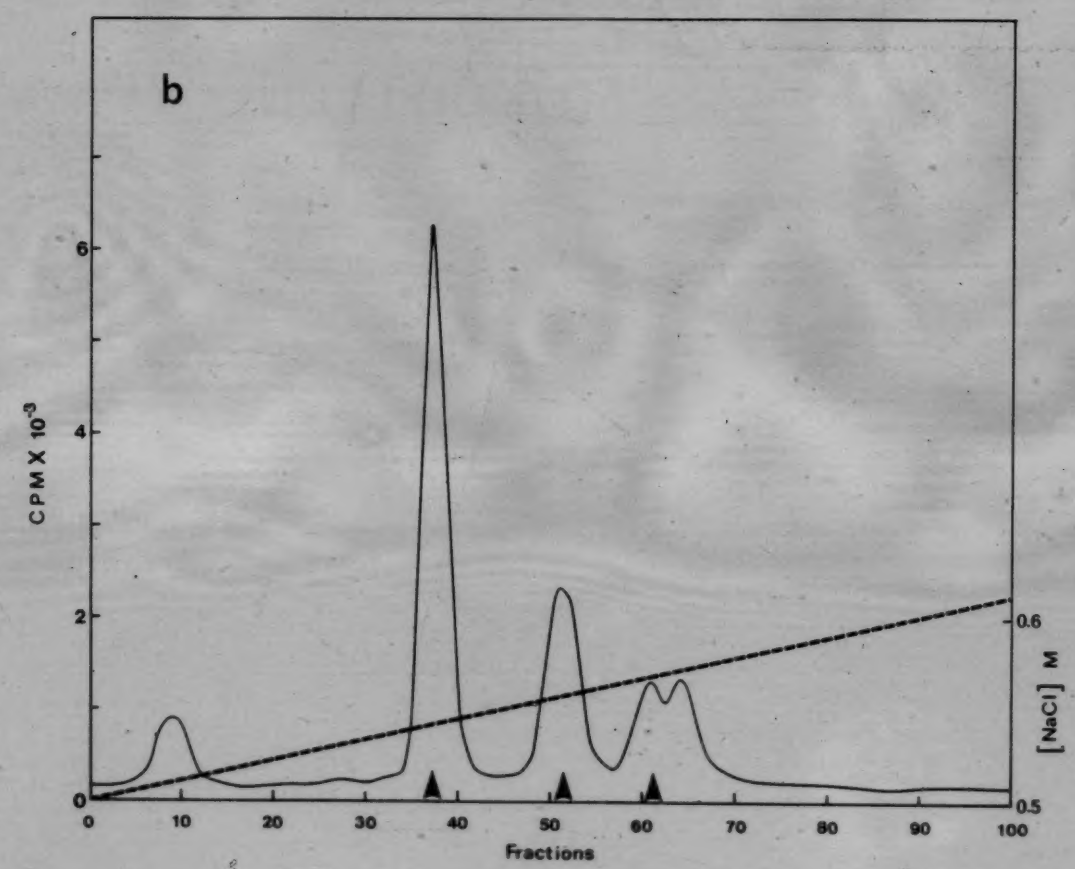
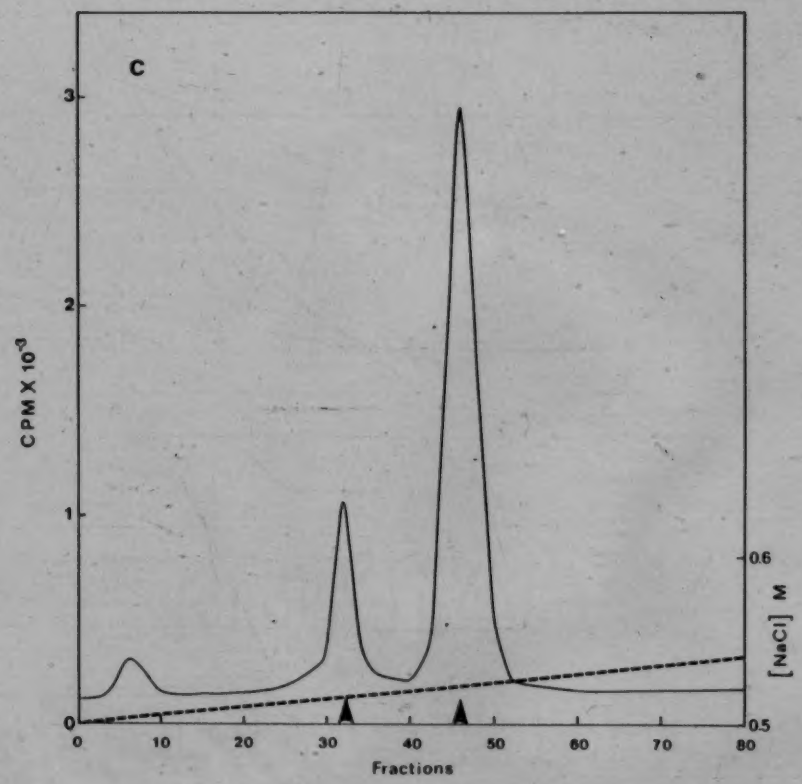
	$_{a}$tRNA^{Phe}	$_{a}$tRNA^{Tyr}	$_{a}$tRNA^{Ser}	$_{a}$tRNA^{Val}	$_{a}$tRNA^{Met}	$_{a}$tRNA^{Leu}	$_{b}$tRNA^{Asp}	$_{b}$tRNA^{Gly}	$_{b}$tRNA^{His}
S288C[c]									
XB109-5B									
[d]760-8A									
760-8B									
760-8C									
760-8D									

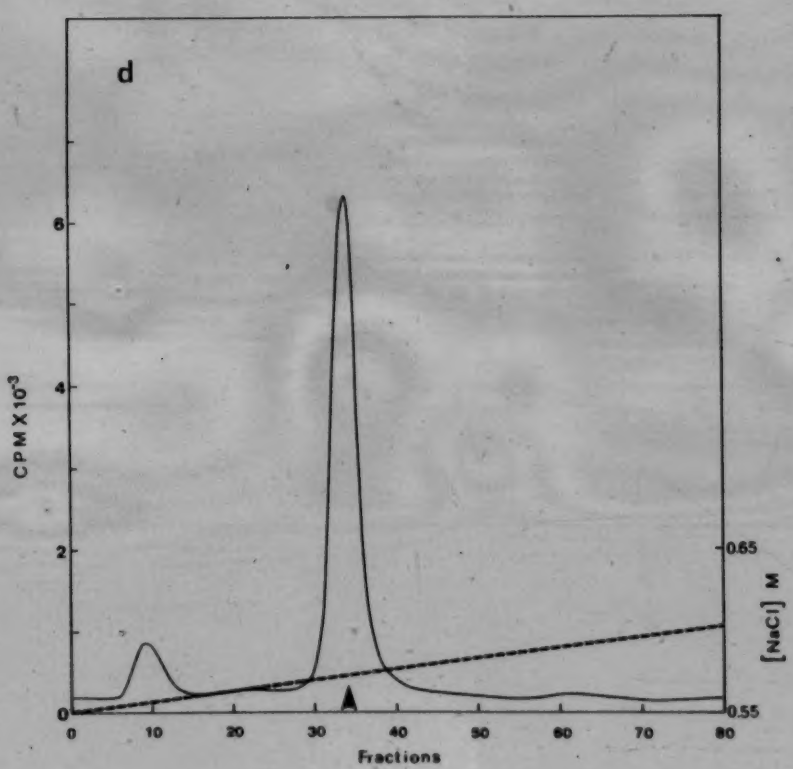
Figure 5

RPC-5 chromatograms of aminoacyl-tRNAs from XB109-5B. Crude tRNA from XB109-5B was aminoacylated with [^{3}H]L-amino acids and chromatographed on RPC-5 as described in Experimental Procedures. The small peak at the beginning of each chromatogram is free amino acid derived from deacylation of the tRNA samples between the time of preparation and application to the RPC-5 column. The arrowheads in each chromatogram indicate the corresponding isoacceptor(s) that was resolved from wild type (S288C). a) $tRNA^{Met}$, b) $tRNA^{Leu}$, c) $tRNA^{Gly}$, d) $tRNA^{His}$.


a
CPM X 10⁻²
15
10
5
0
0.6
0.5
[NaCl] M
0
10
20
30
40
50
60
70
80
90
100
110
120
Fractions


b
CPM X 10⁻³
6
4
2
0
0.6
0.5
[NaCl] M
0
10
20
30
40
50
60
70
80
90
100
Fractions


c
CPM X 10⁻³
3
2
1
0
0.6
0.5
[NaCl] M
0
10
20
30
40
50
60
70
80
Fractions


d
CPM X 10⁻³
6
4
2
0
0.65
0.55
[NaCl] M
0
10
20
30
40
50
60
70
80
Fractions

isoacceptors. Indeed, the detection of mutant acceptors for a tRNA is used by us as an operational definition of *mia* . The six tRNAs which are known to be affected in *mia* strains all show the identical pattern of 2:2 segregation (Table 6). Furthermore, we have now examined a total of ten tetrads for the segregation pattern of *mia* tRNAPhe and all show the expected 2:2 segregation pattern.

Amino Acid Acceptance of Mutant Isoacceptors

Comparisons of chromatograms of particular tRNA species from S288C (or any *MIA*$^+$ strains) with those of *mia* demonstrate that, for any tRNA species affected by *mia* , the wild type acceptor(s) is(are) also always produced. This is suggested by the co-elution of one or more of the isoacceptors from *mia* with the cognate wild type acceptor(s) from *MIA*$^+$ in co-chromatograms (Bell *et al.*, 1977; and this paper). In order to determine whether these mutant tRNAs in *mia* influence the final levels of aminoacylation, we have compared the amino acid acceptance levels of crude tRNA samples extracted from *mia* and *MIA*$^+$ strains. The results for phenylalanine, tyrosine, and glycine acceptances are presented in Table 7. These results indicate that crude tRNAs from *mia* and *MIA*$^+$ strains have the same acceptor levels for a particular amino acid regardless of whether the tRNA species is affected by *mia* or not. In addition, the kinetics of aminoacylation of *mia* tRNAs are similar to *MIA*$^+$ tRNAs. (See Appendices)

Table 7. Amino acid acceptance of bulk tRNA samples from *MIA*$^+$ and *mia*

	pmoles / A_{260} nm		
	Phenylalanine	Tyrosine	Glycine
JB759-5A *MIA*$^+$	50.0	53.3	184
JB759-5B *mia*	50.3	47.9	182

Bulk tRNA was aminoacylated with [^{14}C]L-amino acids in a reaction which contained: 100 mM Tris-Cl(pH 7.5), 20 mM $MgCl_2$, 6 mM ATP (pH 7), 0.6 mM CTP, 100 mM KCl, 10 mM β-mercaptoethanol, 50 µl crude yeast aminoacyl-tRNA synthetase, and ~2 A_{260} nm units of tRNA in a final volume of 200 µl. At timed intervals, 20 µl was spotted on Whatman paper, washed in cold TCA (3 times), ethanol, ether, and then air-dried for liquid scintillation counting. The acceptance levels are plateau values after 5 minutes of reaction.

Production of Mutant Isoacceptors at Different Growth Stages of a *mia* Culture

The determination of growth kinetics was carried out as described in experimental procedures and it was demonstrated that the presence of mutant isoacceptors does not have any adverse effects on the growth rates of *mia* strains. (See Appendices) If mutant isoacceptors were accumulated in stationary cultures only, this would explain the apparent wild type growth kinetics of *mia* strains since, at the active growing stages, all the tRNA molecules would be wild type. In order to characterize this aspect of *mia* more closely, we determined the level of mutant isoacceptors throughout the growth cycle of *mia* cultures.

Cultures of JB759-5B *mia* were harvested at different stages of growth (as indicated by OD_{550}). Crude tRNAs were extracted and RPC-5 chromatograms of Phe-tRNAPhe were obtained for each culture. The relative amounts of each peak in the chromatograms (representing each isoacceptor) were calculated and the data are presented in Table 8. As a comparison, results from JB759-5A *MIA*$^+$ harvested at an early stage of growth and at saturation are also presented. In a standard RPC-5 chromatogram of *mia* Phe-tRNAPhe, three isoacceptors are observed and are named A, B, and C, respectively, in order of elution. Isoacceptor A chromatographs with wild type Phe-tRNAPhe and is assumed to be the wild type acceptor (Bell *et al.*, 1977).

Table 8. Influence of growth stage on the relative amounts of $tRNA^{Phe}$ isoacceptors in *mia* and *MIA*$^+$ cultures

	OD_{550} nm	Relative Amounts of $tRNA^{Phe}$ Isoacceptors		
		A	B	C
JB759-5B *mia*	0.5	0.05	1	1.9
	1.5	0.18	1	1.0
	2	0.25	1	0.8
	5	0.27	1	0.9
	10	0.33	1	0.69
	24 (saturation)	0.44	1	0.38
JB759-6A *MIA*$^+$	2	only isoacceptor A		
	24 (saturation)	only isoacceptor A		

The yeast cultures were grown in liquid YEPD under the standard shaking conditions and the RPC-5 chromatograms were prepared from each culture as described. The relative amounts of isoacceptors A and C are standardized to isoacceptor B since it is often the most abundant isoacceptor.

The results in Table 8 demonstrate that *mia* cultures during early logarithmic stages have a higher proportion of mutant isoacceptors than previously measured at late logarithmic or saturation phases. Indeed, in early stages of growth, isoacceptor A was virtually absent. In contrast, when tRNA was examined from a *MIA*$^+$ strain during an early stage of the culture, only the wild type acceptor was observed. Also, in the *mia* culture there is a coincidental decrease in the relative amount of mutant isoacceptor C with the increasing levels of isoacceptor A during the growth cycle.

In Vivo Pulse-label and Chase Studies on *mia* Cultures

A precursor-product relationship between mutant acceptors and the cognate wild type acceptor tRNA predicts specific alterations in acceptor profiles during pulse-chase experiments. We have attempted to ascertain such changes and present our data below.

A *mia* strain was grown in the presence of [^{3}H]-uracil for a period of nine hours. Transfer RNA was extracted and tRNAPhe was partially purified from bulk tRNA by a BD-cellulose chromatography step. The specific activities of the three tRNAPhe isoacceptors were then determined from RPC-5 chromatograms. The BD-cellulose chromatography step was very effective in eliminating background [^{3}H] counts from our chromatograms, as judged by the co-elution of the [^{3}H] with [^{14}C] Phe-tRNAPhe counts on the subsequent RPC-5

chramotograms. As indicated in Table 9, all three isoacceptors of tRNAPhe are labelled to the same extent during the pulse-label period. After the nine-hour labelling period, the label was removed from the medium and the cells were allowed to grow for another nine hours in the absence of [^{3}H]-uracil. After this chase period, the specific activities of the three isoacceptors of tRNAPhe were remeasured. If the three isoacceptors are independent of each other, then we again expect similar specific activities among them. However, the data in Table 9 indicate that isoacceptor A now has the highest specific activity, followed by isoacceptor B, and then isoacceptor C.

With a shorter label and a longer chase period (6-hour pulse and 12-hour chase), the apparent gradient in specific activities of the three isoacceptors is even more dramatic. By doing just a short pulse-label (with no chase) at any growing stage of the culture, we observed the highest specific activity in isoacceptor C, followed by isoacceptor B, and then isoacceptor A.

Expression of the *mia* Phenotype as Influenced by Temperature

When tRNA is examined from a *MIA*$^+$ strain grown at 37°C (an elevated temperature compared to the usual growing temperature of 30°C), there is evidence that some mutant isoacceptors are accumulated (Figure 6a); albeit in quantities so small that they could easily have been

Table 9. Specific activities of the $tRNA^{Phe}$ isoacceptors from *in vivo* pulse-label and chase experiments.

[a] The [^{3}H]-uracil labelled tRNA samples were partially purified by BD-cellulose chromatography, aminoacylated with [^{14}C]L-phenylalanine and chromatographed on an RPC-5 column. Total [^{3}H] and [^{14}C] cpm for each isoacceptor is obtained from the chromatograms and the specific activities in terms of [^{3}H]cpm / [^{14}C]cpm are calculated. For better illustration of the gradient of specific activities for the three isoacceptors, the relative specific activities are presented after standardizing the actual values to the isoacceptor with the highest specific activity in each case.

[b] The OD_{550} of the cultures during the 9 hr and 6 hr labelling periods are 0.05 to 2, and 0.05 to 0.8, respectively. The chase is then continued until saturation.

[c] The 3 hr labelling culture started at an OD_{550} of 15 and continued until saturation.

	Relative Specific Activities of $tRNA^{Phe}$ Isoacceptors[a]		
	A	B	C
9 hr label[b]	1	0.99	0.96
9 hr label 9 hr chase	1	0.78	0.72
6 hr label 12 hr chase	1	0.63	0.28
3 hr label[c]	-	0.68	1

overlooked previously (Bell *et al.*, 1978). When we examine tRNA from a *mia* strain grown at the elevated temperature (37°C), the wild type acceptor is virtually absent (Figure 6b). However, when the tRNAPhe profile of a *mia* strain grown at 23°C is examined, a wild type phenotype is resolved (results not shown). It appears, then, that *mia* has no effect on cultures grown at 23°C, but has an increasingly mutant phenotype as we examine tRNAs from cultures grown at progressively elevated temperatures.

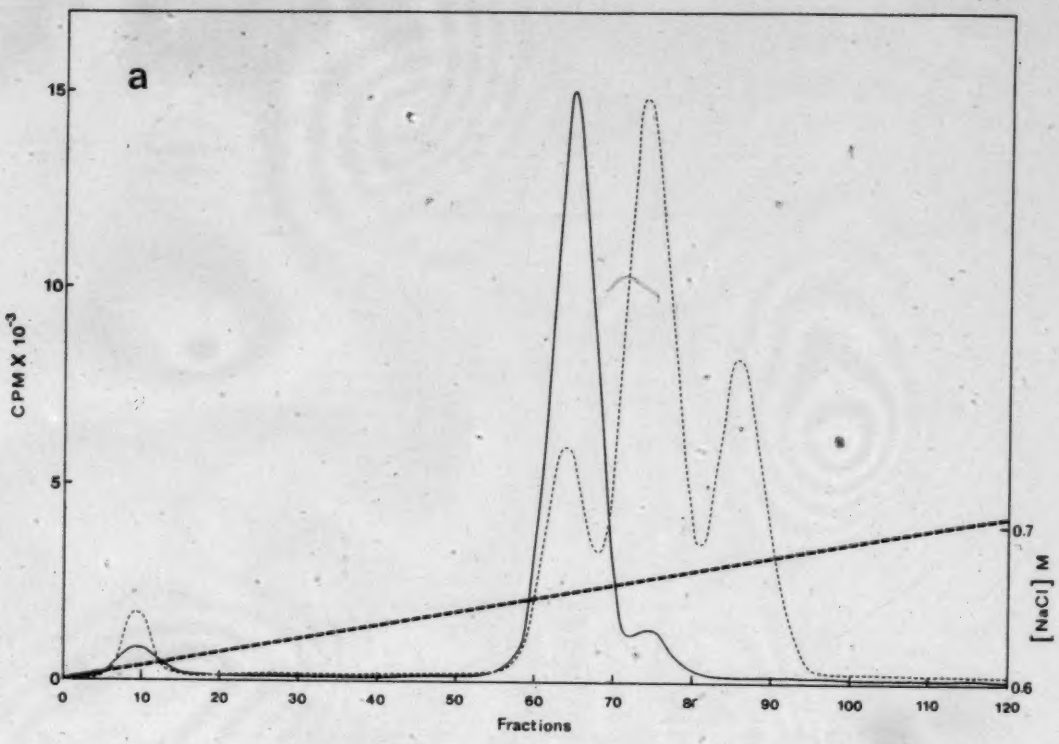
Mutant Isoacceptors Are Not Deficient in N^2-dimethylguanine

Transfer RNAs from yeast strain D38 (containing *trm1*) lack N^2-dimethylguanine due to an inactive enzyme for the methylation of guanine to N^2-dimethylguanine (Phillips and Kjellin-Straby, 1967). Since it is possible that mutant isoacceptors which accumulate in *mia* strains are deficient in certain base modifications, the tRNAs from the *trm1* strain were examined and compared to the mutant isoacceptors from *mia* . The published sequence of yeast tRNAPhe (in Barrell and Clark, 1974) shows that there is one molecule of N^2-dimethylguanine at residue position 26 of the mature wild type molecules, thus making tRNAPhe an appropriate species for these comparisons.

An RPC-5 co-chromatogram of the Phe-tRNAPhe from strain D38 and wild type (S288C) is presented in Figure 7a, and this shows that only one acceptor of tRNAPhe is resolved for

Figure 6

RPC-5 co-chromatograms portraying the Phe-tRNA^{Phe} profiles of JB759-5A *MIA*$^{+}$ and JB759-5B *mia* grown at 37^{o}C, together with the standard *mia* phenotype of JB759-5B grown at 30^{o}C. a) ——— represents [^{3}H]L-phenylalanyl-tRNA^{Phe} from JB759-5A *MIA*$^{+}$ grown at 37^{o}C, and ----- represents [^{14}C]L-phenylalanyl-tRNA^{Phe} from JB759-5B *mia* grown at 30^{o}C. b) ——— represents [^{3}H]L-phenylalanyl-tRNA^{Phe} from JB759-5B *mia* grown at 37^{o}C, and ----- represents [^{14}C]L-phenylalanyl-tRNA^{Phe} from JB759-5B *mia* grown at 30^{o}C.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CPM X 10⁻³
15
10
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Fractions
0.7
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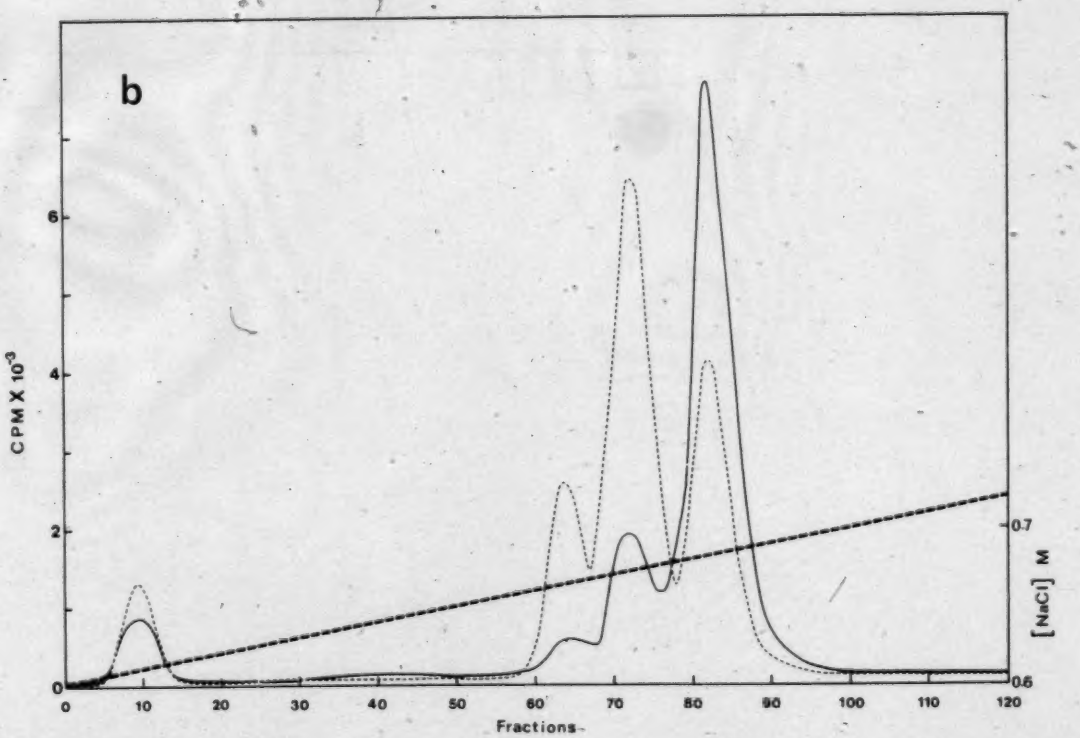
[NaCl] M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Fractions
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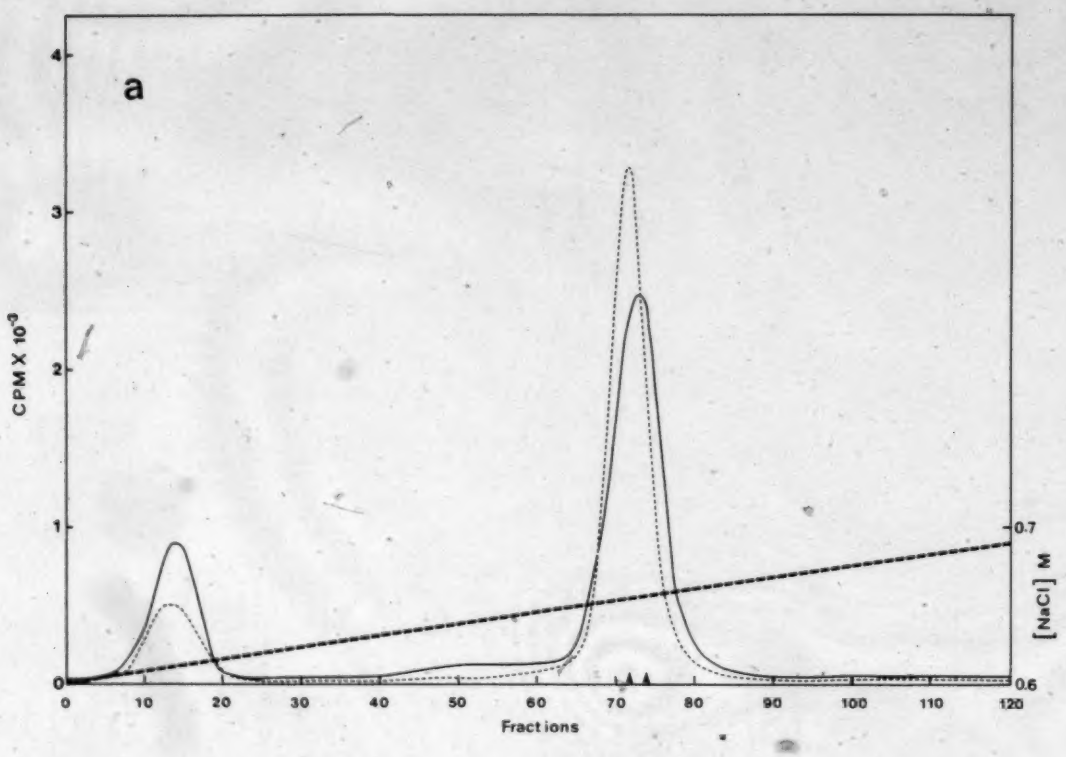
[NaCl] M

D38. This acceptor is apparently not the wild type tRNAPhe, by virtue of its slightly altered chromatographic position. A single acceptor is also resolved for tRNATyr from D38; however, this acceptor is shifted more (8 fractions) from the wild type position (Figure 7b). Similar chromatographic conditions were used for the two tRNA species. This difference in the alteration of chromatographic positions for the two species of tRNA from their wild type positions probably reflects the different tertiary structures of the two tRNA species and their interactions with the RPC-5 separating material, since both tRNAPhe and tRNATyr have a single N^2-dimethylguanine at approximately the same residue position (Barrell and Clark, 1974).

In order to examine phenotypic interactions between *mia* and *trm1*, haploid double mutant strains were identified by analysing the tRNA profiles of tetrads sporulated from the diploid constructed between *trm1* and *mia* . The segregation of auxotrophic markers in these tetrads was also examined in order to demonstrate that *bona fide* tetrads were analysed. From the tRNAPhe profile of a *mia trm1* mutant from one of these tetrads (Figure 8), it appears that *trm1* interacts with all three *mia* tRNAPhe isoacceptors (all three acceptors are displaced relative to the marker tRNAPhe from *mia*) and there is no epistasis of one mutant over the other. Co-chromatograms of Tyr-tRNATyr from the double mutant strain and *mia* also show the characteristic altered chromatographic positions for N^2-dimethylguanine deficient

Figure 7

RPC-5 co-chromatograms portraying the Phe-tRNA^{Phe} and Tyr-tRNA^{Tyr} profiles of strain D38 compared to wild type S288C. a) ——— represents [^{3}H]L-phenylalanyl-tRNA^{Phe} from D38, and ----- represents [^{14}C]L-phenylalanyl-tRNA^{Phe} from S288C. Phe-tRNA^{Phe} from D38 is shifted 2 fractions from Phe-tRNA^{Phe} of S288C as indicated by the arrowheads. b) ——— represents [^{3}H]L-tyrosyl-tRNA^{Tyr} from D38, and ----- represents [^{14}C]L-tyrosyl-tRNA^{Tyr} from S288C. Tyr-tRNA^{Tyr} from D38 is shifted 8 fractions from Tyr-tRNA^{Tyr} of S288C. Similar chromatographic conditions were used for the two tRNA species.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Fractions
0.6
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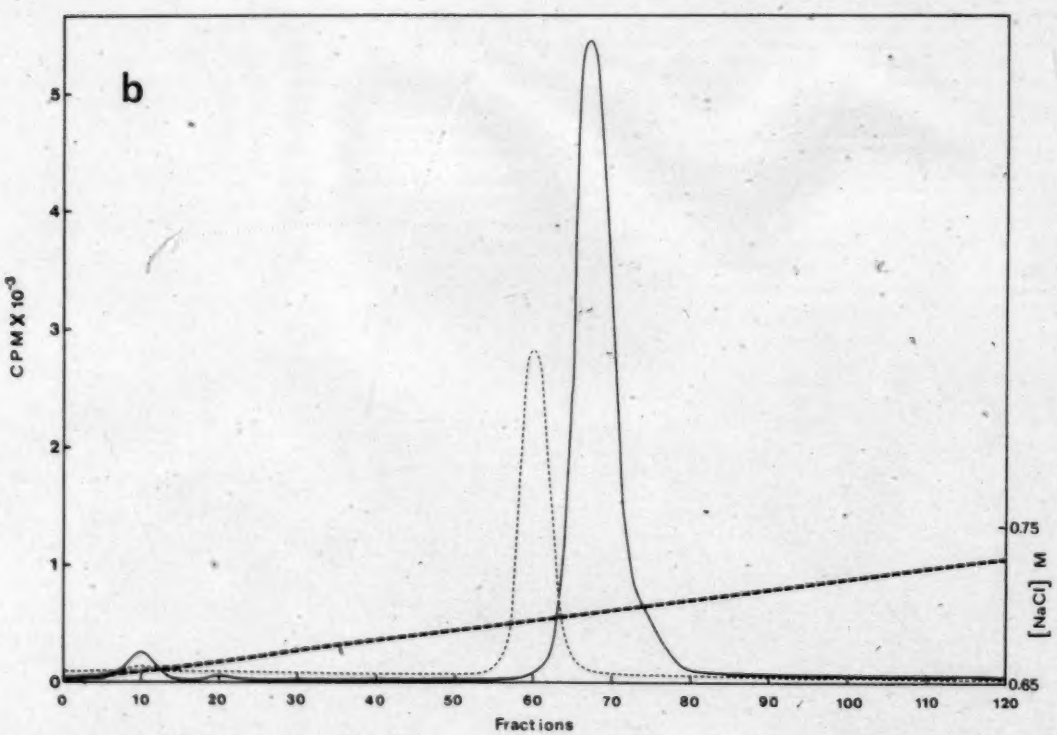
[NaCl] M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100
110
120
Fractions
0.65
0.75
[NaCl] M

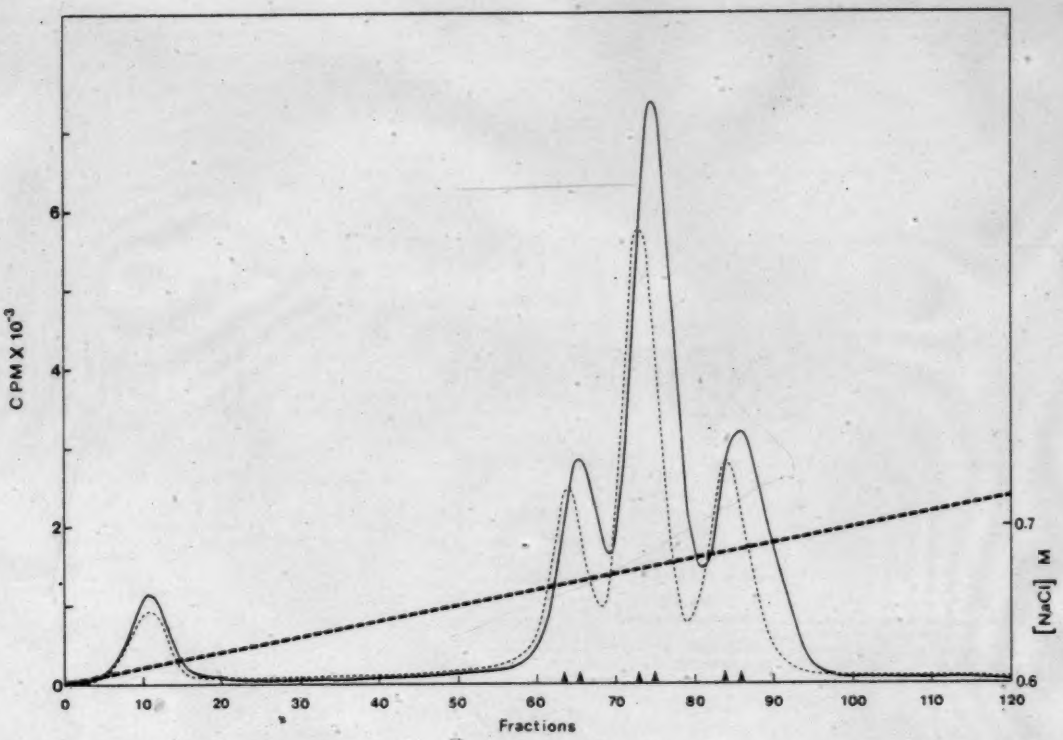

Figure 8

RPC-5 co-chromatogram portraying the Phe-tRNA^{Phe} profiles of the double mutant RL101-5B together with JB759-5B *mia*. ——— represents [^{3}H]L-phenylalanyl-tRNA^{Phe} from RL101-5B, and ----- represents [^{14}C]L-phenylalanyl-tRNA^{Phe} from JB759-5B *mia*. All three isoacceptors of tRNA^{Phe} from RL101-5B are shifted from their normal positions. This is characteristic of N^{2}-dimethylguanine-less tRNAs (see Figure 7). RL101-5B is a double mutant of *mia* and *trm1*.

tRNATyr for all four tyrosine isoacceptors of *mia* (data not shown).

Methyl Acceptance Levels of *mia* Transfer RNA

The relative ability of bulk tRNAs from *mia* and *MIA*$^+$ to accept methyl groups from S-[methyl-^{14}C]-adenosylmethionine was determined. A crude methylase preparation was prepared from wild type yeast (S288C) using the method of Björk and Svensson (1969). High acceptance levels were observed when this crude enzyme preparation was used on bulk tRNA from strain D38, which is a good substrate for methylation due to the absence of N^2-dimethylguanine (Phillips and Kjellin-Stråby, 1967). Bulk tRNA from a *mia* culture consistently demonstrates higher methyl group acceptances than control tRNA from *MIA*$^+$ cultures, and Figure 9 shows a typical result. However, it should be noted that the level of methyl group acceptance by *mia* tRNA, although considerable when compared to *MIA*$^+$ tRNA, is still small by comparison with that of *trm1* tRNA. The possible significance of this result will be discussed below.

Discussion

Comparisons of RPC-5 chromatograms of nine different tRNA species from XB109-5B and wild type, together with the segregation analysis of the respective tRNA profiles in tetrad JB760-8A, B, C and D, indicate that a single mutation is responsible for the production of mutant isoacceptors for

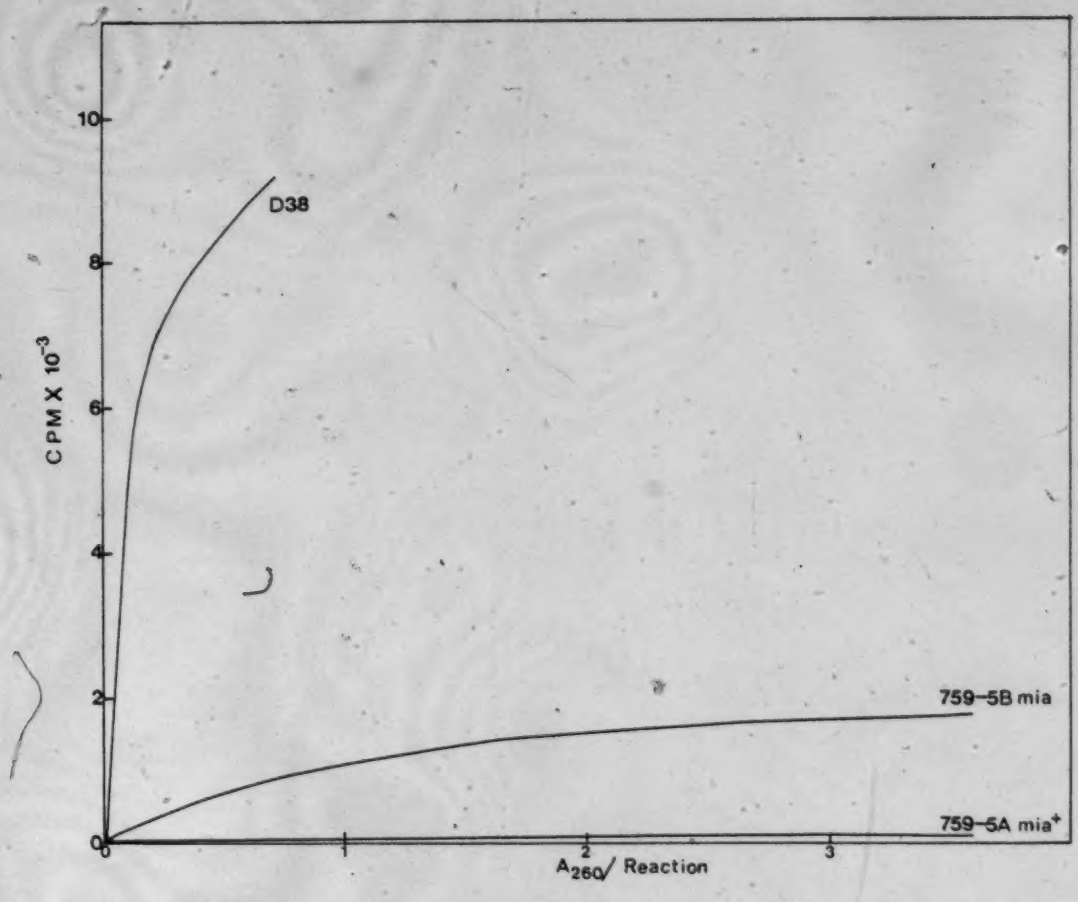

Figure 9.

Acceptance levels of [^{14}C]-methyl for bulk *MIA*$^{+}$ and *mia* tRNAs. The assays are as described in Experimental Procedures using S-[methyl-^{14}C]-adenosylmethionine as the methyl donor. Bulk D38 tRNA methyl acceptance serves as a control since the tRNAs in this case are known to be substrates for methylation by yeast enzymes.

the tRNA species examined. This is an extension of the data presented in Bell *et al.* (1977).

The similar levels of amino acid acceptance using bulk *mia* and *MIA*$^+$ tRNA demonstrates that mutant isoacceptors are produced at the expense of normal levels of wild type tRNAs. This eliminates the possibilities that mutant isoacceptors are merely the result of non-specific aminoacylation of different tRNA species or due to redundant production of particular tRNA species, which are then not fully processed. We have also observed that the same mutant isoacceptors are present in spontaneous petite strains isolated from *mia* indicating that mutant isoacceptors do not have a mitochondrial origin (see appendices); this result is in agreement with the segregation data.

It is apparent from RPC-5 chromatograms that a major portion of the tRNA pool in *mia* cells is usually in the mutant form(s). However, this does not seem to have any adverse effects on cells harbouring this mutation since the growth kinetics of *mia* and *MIA*$^+$ cultures are identical and both achieve the same levels of saturation. In fact, *mia* cells have no detectable phenotype other than the altered tRNA molecules. The observation that *mia* cultures have even smaller amounts of wild type tRNA at early stages of growth, and almost undetectable amounts of wild type tRNA when grown at 37°C, further suggests that the mutant isoacceptors are biologically active molecules. The occurrence of mutant isoaccepting tRNAs with the essential biological activities

intact is not unexpected since tRNAs deficient in N^2-dimethylguanine do not seem to impair the growth of the cells in strain D38. Furthermore, Laten *et al.* (1978) reported that the *mod5-1* mutant in *S. cerevisiae*, which is known to lack isopentenyladenosine in its tRNAs, grows as well as *MOD5*$^+$ strains in a genetic background lacking a Class I suppressor. Preliminary results from *in vitro* translation of poly-U using a wheat germ S-30 extract and a purified preparation of tRNAPhe from *mia* (containing all three isoacceptors) versus *MIA*$^+$ tRNAPhe are also consistent with the hypothesis that the *mia* tRNAs are fully functional in protein synthesis (unpublished results).

The most likely explanation for the pleiotropic effect of *mia* is that it is a mutation in some modification step(s) in the maturation of tRNA molecules, but a comparison of the primary sequences (Barrell and Clark, 1974; Gauss *et al.*, 1979) of the tRNA species examined fails to implicate any particular deficiency to account for the results obtained. The elution of mutant isoacceptors during RPC-5 chromatography is always at a higher salt concentration than the cognate wild type acceptor. Interestingly, those tRNAs which are known to be undermethylated (i.e., some tRNAs in D38) also elute at a higher salt concentration than the comparative wild type tRNAs. The methylation results reported here are tantalizing in this regard. These may be misleading, or it could be that our extraction or assay procedures are sub-optimal for the specific methylase

activity we are seeking and that is why the acceptance levels of *mia* compare unfavourably to those of *trm1* . Moreover, the chromatographic data on the double mutant of *trm1* and *mia* strongly indicate that if the *mia* mutation results in undermethylation, it is not due to a lesion in the gene encoding the enzyme to make N^2-dimethylguanine. We will pursue trying to optimize the methylation conditions. In addition, further biochemical studies are in progress to determine the nucleoside compositions of 'fingerprint' fragments from highly purified tRNAPhe isoacceptors, and comparing them with those of wild type molecules; thus providing a direct test for undermethylation.

It has been reported that a number of yeast tRNA genes have intervening sequences (Goodman *et al.*, 1977; Valenzuela *et al.*, 1978; Etcheverry *et al.*, 1979; Ogden *et al.*, 1979) and that mutants which accumulate pre-tRNA molecules with mature 5' and 3' termini, but which still contain the intervening sequences, have been reported (Hopper *et al.*, 1978; Hopper *et al.*, 1980). We feel that *mia* is not defective in the splicing of intervening sequences since mutant isoacceptors are aminoacylated normally and appear to be biologically active molecules, while the pre-tRNA molecules which contain the intervening sequence could not be aminoacylated (O'Farrell *et al.*, 1978). However, we do notice an interesting correlation between the tRNA species affected by *mia* and the tRNA species that have intervening sequences in their genes. Of the tRNA species affected by

mia (Table 6), tRNAPhe, tRNATyr, tRNASerUCG, and tRNA,Leu have been reported to have intervening sequences in their genes, while tRNAAsp does not have an intervening sequence and is not affected by *mia* . This may merely be a coincidence, but we are analysing more tRNA species from our *mia* and *MIA*$^+$ strains to determine if the correlation holds. It is possible that the defect in *mia* is involved in the maturation of a subset of tRNA species which have intervening sequences in their primary transcripts.

Inspection of RPC-5 chromatograms of *mia* and *MIA*$^+$ tRNAs reveals that in nearly all cases where mutant isoacceptors are produced, the wild type acceptors are also present. Results from the *in vivo* pulse-label and chase experiments, which demonstrate that mutant isoacceptors can be converted to the wild type molecules, suggest that mutant isoacceptors may be precursors to the wild type molecules. However, other hypotheses are certainly tenable. Our working hypothesis is that mutant isoacceptors probably accumulate due to a defective modification process and represent undermodified molecules which are biologically active. The mutant profile of *mia* grown at 37°C supports this notion. At this elevated temperature it is possible that the modification step affected by *mia* is even more impaired. A remaining puzzle is the failure to obtain complete *in vivo* conversion of the putative precursor forms to the mature form(s) in late stationary cultures where, one might suppose, a supposedly defective enzyme could be able to handle the reduced amount

of precursor substrate. We have no explanation for this, but perhaps the enzyme itself is no longer produced, or becomes inactive, at the late stages of culture growth.

Finally, there is some recent evidence for a particular sequence of maturation in yeast (Melton *et al.*, 1980). Our results from the double mutant of *mia* and *trm1*, which produces mutant isoaccepting tRNAs that are also missing N^2-dimethylguanine, suggest that at least some of the steps are independent of each other if we assume that *mia* is actually a type of processing mutation.

Acknowledgements

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Note Added in Proof

Nucleoside composition analyses of *mia* tRNAPhe isoacceptors indicate that *mia* strains are defective in the production of some of the dihydrouridine moieties normally found in tRNAs.

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V. Dihydrouridine-deficient tRNAs In *SACCHAROMYCES CEREVISIAE*

* Submitted for publication

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T6G 2E9

Abstract

A mutation in *Saccharomyces cerevisiae*, designated *mia*, is responsible for the production of mutant isoaccepting tRNA molecules for several of its tRNA species. The *mia* isoacceptors of tRNAPhe and one of the mutant isoacceptors of tRNATyr were highly purified for nucleoside composition analyses. The data indicate that the mutant isoacceptors are lacking some of the dihydrouridine moieties. This is consistent with our previous hypothesis that the mutant isoacceptors were accumulated due to a defect in a modification process [Lo, R.Y.C. & Bell, J.B. (1981) *Current Genetics* 3, 73-82]. Data from *in vitro* poly-U translation experiments also support the previous results suggesting *in vivo* biological activity of these mutant tRNAs.

Introduction

The primary transcripts of tRNA genes are precursor molecules which must undergo several processing steps to produce the mature tRNAs. These processing steps involve both size alterations and nucleoside modifications (1-4), and require the action of specific enzymes (5, 6). Such interesting problems as the temporal sequence of the nucleoside modifications and the functions of these modified nucleosides in the mature tRNAs remain largely unsolved (6, 7) although data are accumulating (6-8). To solve such problems it will be useful to study mutants defective in tRNA nucleoside modifications.

Apparently, tRNAs lacking particular nucleoside modifications may still function normally during protein synthesis (9-12) and this makes it very difficult to isolate mutants defective in these modification processes. In yeast, only a few examples of such mutants have been reported (11,12 and A. Hopper, personal communication). The *mia* mutation in *Saccharomyces cerevisiae* is responsible for the production of aberrant isoaccepting forms for several tRNA species and this mutant has been partially characterized (13-15). In this paper we present data that identify the molecular lesion in the mutant acceptor tRNAs. Results from nucleoside composition analyses of the purified isoacceptors of *mia* tRNAPhe and one mutant acceptor of tRNATyr indicate that the tRNAs affected by the *mia* mutation are missing some of the dihydrouridine modifications found in the cognate

wild type tRNA molecules.

Materials and Methods

Yeast Strains and Cultures

The yeast strains S288C (wild type), JB759-5A *MIA*$^{+}$, and JB759-5B *mia* , as well as the liquid YEPD media and the growth conditions are as previously described (13).

Preparation of tRNA and Aminoacyl-tRNA Ligase

Crude tRNAs were prepared from yeast cells by phenol extraction and DEAE-cellulose chromatography (16). Crude aminoacyl-tRNA ligases were prepared as described by Nishimura *et al*. (17) with the following modifications: Wild type cells were disrupted by 3 passages through a French pressure cell at 17,000 psi; and 0.2 volumes of a 5% w/v streptomycin sulfate solution were added to the extract before the centrifugation step.

Purification of tRNAPhe and tRNATyr Isoacceptors

Crude tRNAs were deacylated in 1 M Tris-Cl (pH 8) at 37°C for 2 hours, recovered by ethanol precipitation and fractionated by BD-cellulose chromatography as described by Wimmer *et al*. (18). Five ul aliquots of each fraction were assayed for phenylalanine or tyrosine acceptance in a 50 ul reaction at a final concentration of 120 mM Tris-Cl (pH 7.5), 22 mM $MgCl_2$, 7.5 mM ATP, 0.75 mM CTP, 120 mM KCl, 12 mM β-mercaptoethanol, 5-10 uM amino acids (200 Ci/mol and

1000 Ci/mol for [^{14}C] or [^{3}H] amino acids, respectively) and containing 15 ul crude yeast aminoacyl-tRNA ligases. The reactions were carried out at 37°C for 10 minutes after which 40 ul were removed, TCA precipated, washed and dried for liquid scintillation counting.

Partially purified tRNA samples from the BD-cellulose chromatography step were then fractionated by RPC-5 chromatography (19) in 0.05M Tris-Cl (pH 7.5), 0.01M $MgCl_2$, and 0.001M EDTA using a linear gradient from 0.5 to 1M NaCl. Transfer RNAPhe and tRNATyr were recovered after assaying the fractions for the amino acid acceptor activity as above.

To purify the tRNAs further, and also to separate the various isoacceptors, the tRNAs were next aminoacylated and chromatographed on RPC-5 using an acetate buffer at pH 4.5 (13). The different isoacceptors were recovered separately from the relevant fractions, upon completion of the RPC-5 chromatogram. To achieve greater purity for each of the tRNAPhe isoacceptors, a third RPC-5 chromatographic separation was done on the isoacceptors individually. In the case of the isoacceptor D of tRNATyr, no further chromatography was done, since absolute purity of this isoacceptor was not necessary (see Results).

The tRNA samples were tested for purity by assaying their amino acid acceptor activities at each step. The final amino acid acceptances of the purified isoacceptors were all greater than 1200 pmole/A_{260} nm unit.

For analytical RPC-5 chromatography, the tRNAs were aminoacylated with radioactive amino acids as described previously (15). The purified tRNAs were reacted for only 3 minutes due to their very rapid aminoacylation. Since <0.1 A_{260} nm units of purified tRNAs were used in the reactions, 10 A_{260} nm units of carrier tRNA were added to increase the recovery of the aminoacylated tRNAs. The carrier tRNA was added after the reactions were stopped by the addition of Na acetate (pH 4.5). RPC-5 chromatography of aminoacylated tRNAs was carried out as in Bell *et al.* (13). The salt gradients for Phe-tRNAPhe and Tyr-tRNATyr were 0.6-0.9M and 0.6-0.8M, respectively.

Polyacrylamide-urea Gel Electrophoresis

20% polyacrylamide gels (acrylamide:bisacrylamide 39:1) containing 7M urea were prepared according to Peacock and Dingman (20) and cast in a vertical slab gel apparatus (15 x 12 x 0.15 cm) from Aqueboque. The tRNA samples, mixed with a dye solution (50% sucrose, 7 M urea, 0.1% xylene cyanol FF), were loaded on the gels and electrophoresed for 16 hours at 150 V. The gels were then fixed for 15 minutes in 1N acetic acid and stained in 0.05% methylene blue in 0.2M Na acetate (pH 4.6) for 20 minutes. Destaining was acheived in distilled water.

Analysis of the Nucleoside Composition of tRNA Isoacceptors

The nucleoside compositions of purified tRNA isoacceptors were analysed by the tritium derivative method described by Randerath *et al.* (21) [^{3}H] KBH_4 (3.1 Ci/mmole), and thin layer cellulose sheets were from Amersham and Eastman, respectively. The fluorograms were exposed for 2-4 days on Kodak XRP-1 film at -60°C, after which the films were developed and the spots were quantitated by liquid scintillation counting.

In Vitro Poly-U Translation

An S-30 extract was prepared from wheat germ as in Roberts and Paterson (22) except that the pre-incubation step was omitted. The translation assays were also set up as described, with the following modifications: The assays were in a final volume of 100 ul and contained, in addition to the described ingredients (22), 15 mM Mg acetate, 60 mM NH_4Cl and 20 mM KCl. Poly-U and [^{3}H] L-phenylalanine at 2500 Ci/mole (New England Nuclear) were added to start the reactions, after the rest of the ingredients and 0.1 ug of a purified tRNAPhe isoacceptor were pre-incubated at 30°C for 10 minutes. The reactions were carried out at 30°C for 1 hour, with 10 ul samples removed at timed intervals.

Results

Purification of tRNA Isoacceptors

It was necessary to isolate the isoacceptors for nucleoside analyses in order to characterize the actual molecular lesion produced by *mia* . The three isoacceptors of *mia* tRNAPhe as well as the tRNAPhe from *MIA*$^+$ (13) were individually purified as described in Materials and Methods. The final phenylalanine acceptance levels of the purified tRNAs are listed in Table 10, and an RPC-5 chromatogram of each purified isoacceptor from *mia* is presented in Fig. 10. These levels monitor the purity of the tRNA samples with respect to contamination by other tRNA species while the RPC-5 chromatograms monitor the purity with respect to contamination by the other isoacceptors. The data in Table 10 also support previous results which show that there is no deficiency in the ability of mutant acceptors to be aminoacylated (15).

In addition to the tRNAPhe isoacceptors, one of the four isoacceptors of tRNATyr from *mia* and the wild type acceptor of tRNATyr from *MIA*$^+$ (14) were also purified for nucleoside analysis. Only the last isoacceptor to elute from RPC-5 (called isoacceptor D) was purified, since it was expected to differ most from wild type tRNATyr. It was not necessary to obtain completely purified isoacceptor D since contaminating tRNATyr isoacceptors are also mutant and we were not looking for the absolute quantitative differences between isoacceptor D and wild type tRNATyr (see

Table 10. Amino acid acceptance levels of the purified tRNA isoacceptors

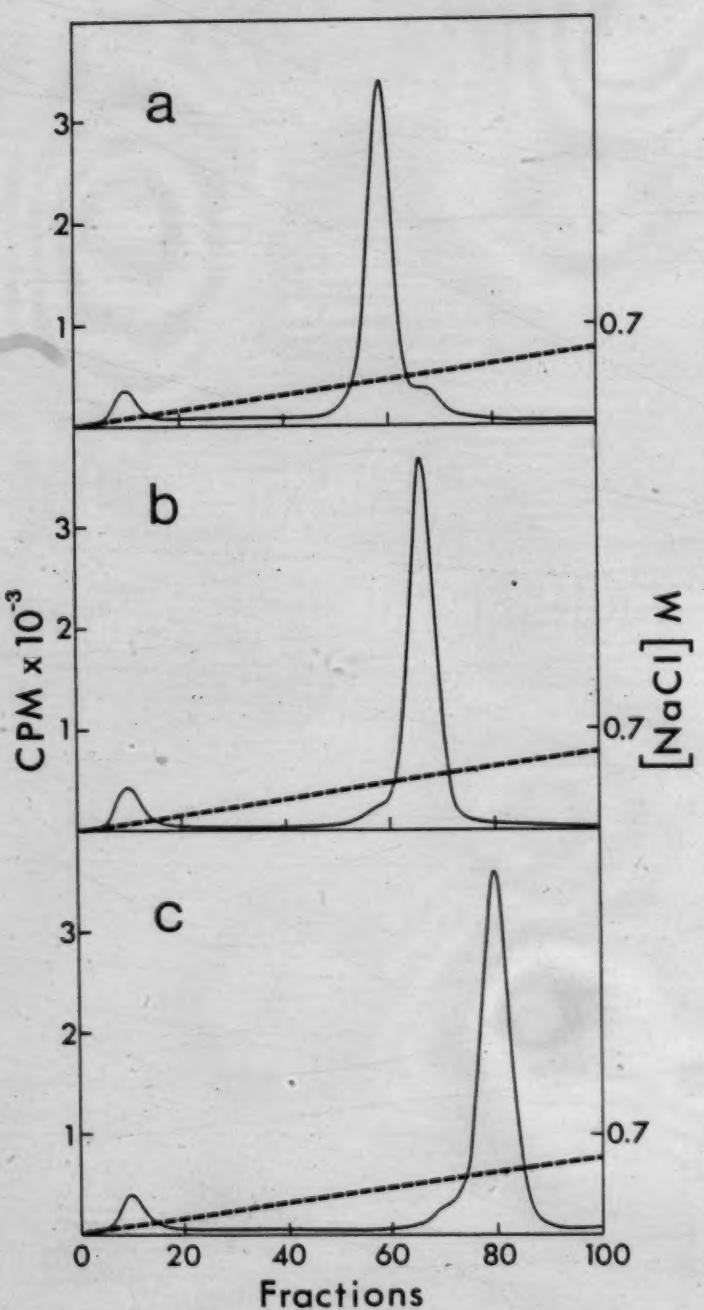
$tRNA^{Phe}$ isoacceptors	pmoles phenylalanine/A_{260} nm
MIA$^+$	1287
mia isoacceptor A	1257
mia isoacceptor B	1542
mia isoacceptor C	1589

$tRNA^{Tyr}$ isoacceptors	pmoles tyrosine/A_{260} nm
MIA$^+$	1437
mia isoacceptor D	1565

The aminoacylation assays were carried out for 3 min as described in Lo & Bell (15). [^{14}C]-phenylalanine and [^{14}C]-tyrosine were present at a concentration of 4.75 μM and 15 μM, and final specific activities of 500 Ci/mole and 100 Ci/mole, respectively.

Figure 10.

RPC-5 chromatograms of the purified isoacceptors of $tRNA^{Phe}$ from JB759-5B *mia*. The three isoacceptors of $tRNA^{Phe}$, a) isoacceptor A, b) isoacceptor B, and c) isoacceptor C were named according to their elution order during RPC-5 chromatography of bulk *mia* tRNA (13). The small peak at the beginning of each chromatogram is free amino acid derived from deacylation of the tRNA samples between the time of preparation and application to the RPC-5 columns.


a
b
c
3
2
1
0.7
CPM x 10^-3
[NaCl] M
0
20
40
60
80
100
Fractions

Discussion). The tyrosine acceptor activities of the purified tRNATyr samples are also presented in Table 10, while an RPC-5 co-chromatogram of tRNATyr isoacceptor D and total *mia* tRNATyr is presented in Fig. 11.

Sizing of tRNA Molecules

The purified tRNAs were analysed by polyacrylamide-urea gel electrophoresis to determine if the mutant isoacceptors are the same length as the cognate wild type molecules. Under the denaturation conditions employed for electrophoresis (7M urea), the molecules are resolved primarily according to their size differences. The lengths of tRNAPhe and tRNATyr are 76 and 78 nucleotides, respectively (23). It can be seen from Fig. 12 that these two species of tRNA are resolved. Further, the four purified tRNAPhe isoacceptors all migrate to one position and the two purified tRNATyr isoacceptors both migrate to another position. This suggests that there are no size and/or configuration differences between the mutant tRNA isoacceptors and the respective wild type molecules. It is also apparent from Fig. 12 that the purified tRNA isoacceptors are free of any major contaminating materials.

Translational Activity of Isoacceptors *In Vitro*

The purified tRNAPhe isoacceptors were assayed for the ability to mediate the production of poly-phenylalanine in an *in vitro* translation system using poly-U as the template.

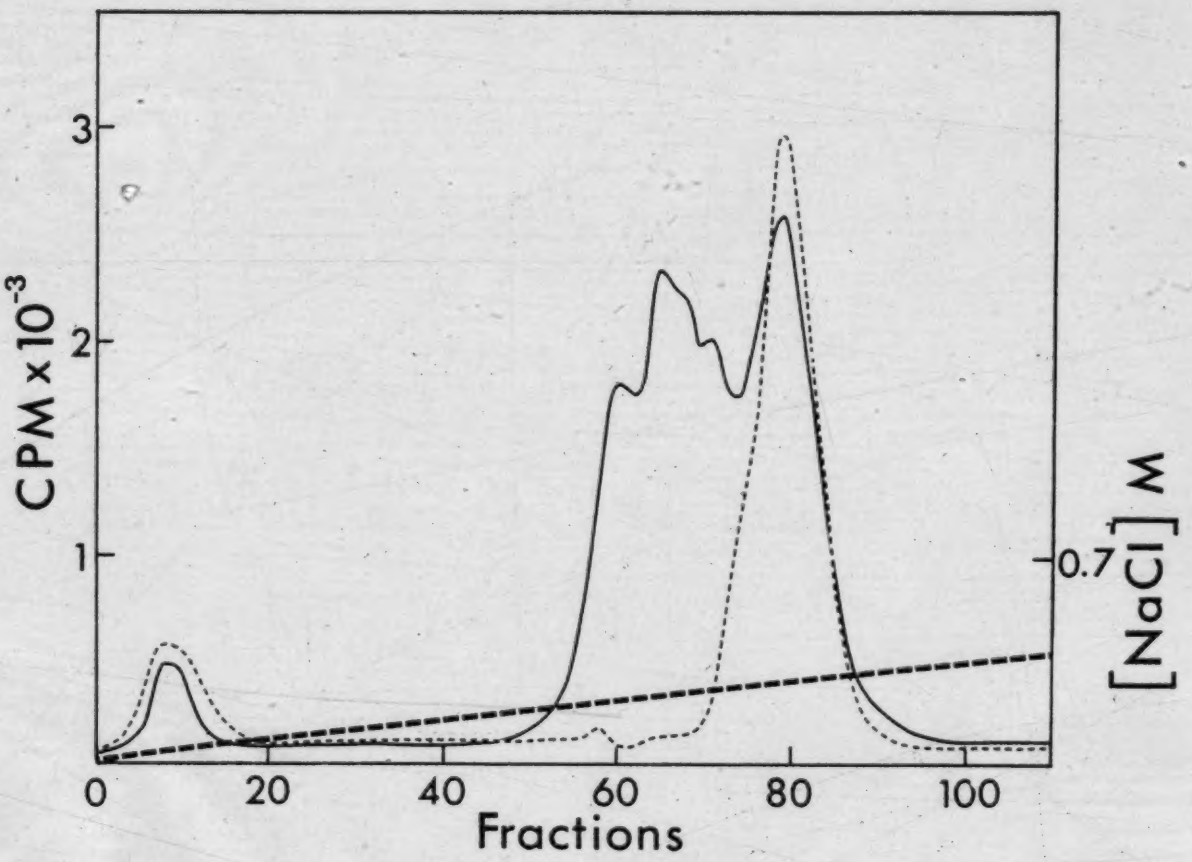


Figure 11

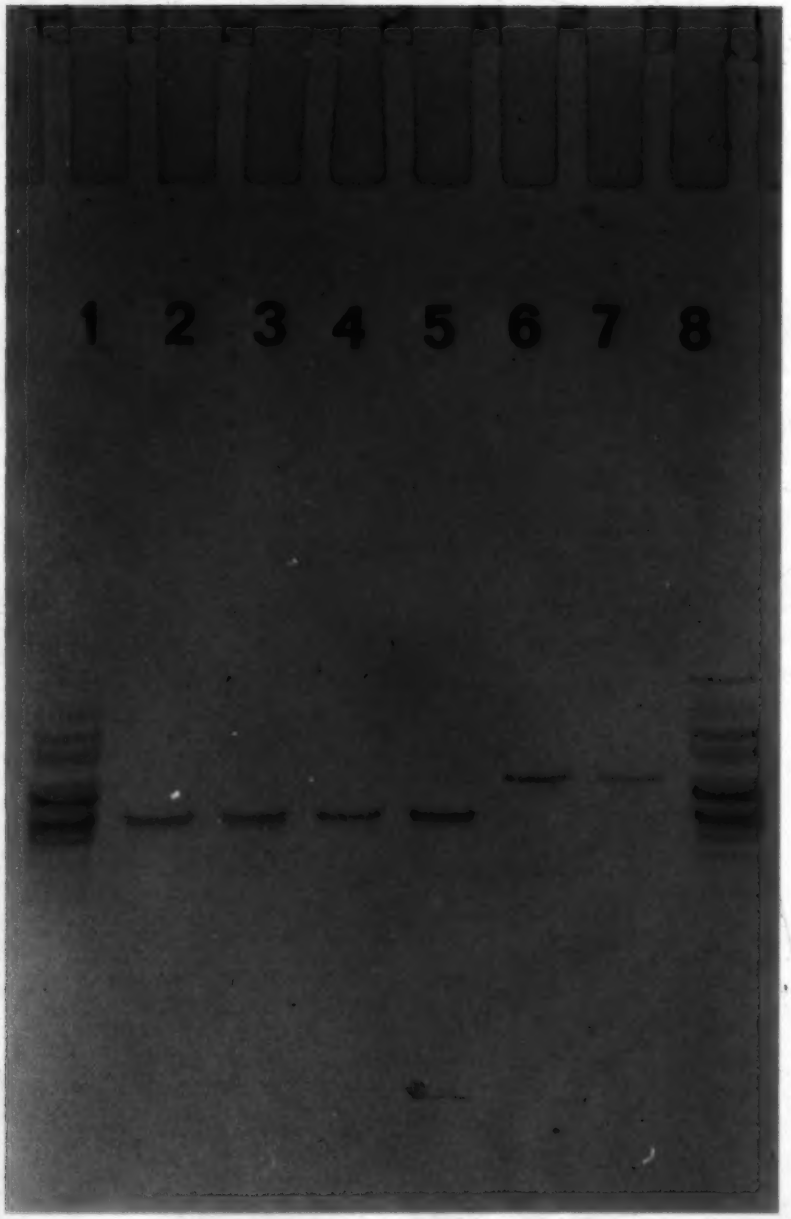
RPC-5 co-chromatogram of the purified isoacceptor D of *mia* $tRNA^{Tyr}$ together with bulk *mia* $tRNA^{Tyr}$ from JB759-5B *mia*.

..... represents [^{14}C]-tyrosyl-$tRNA^{Tyr}$ of purified *mia* $tRNA^{Tyr}$ isoacceptor D.

——— represents [^{3}H]-tyrosyl-$tRNA^{Tyr}$ of bulk $tRNA^{Tyr}$ from JB759-5B *mia*.

Figure 12

A 20% polyacrylamide-urea gel after electrophoresis of tRNA samples. Slots 1 and 8 contain 3 µg of bulk tRNA from wild type S288C and JB759-5B *mia*, respectively. About 0.3 µg of purified tRNA samples were applied to the remaining slots: 2) wild type $tRNA^{Phe}$, 3) *mia* $tRNA^{Phe}$ isoacceptor A, 4) *mia* $tRNA^{Phe}$ isoacceptor B, 5) *mia* $tRNA^{Phe}$ isoacceptor C, 6) wild type $tRNA^{Tyr}$, and 7) *mia* $tRNA^{Tyr}$ isoacceptor D.


1 2 3 4 5 6 7 8

It can be seen in Fig. 13 that there are no differences in the kinetics of tRNA-dependent poly-phenylalanine formation when each purified isoacceptor is used individually in the assays.

Nucleoside Composition Analysis

The nucleoside compositions of each of the purified tRNA isoacceptors were quantitatively determined as described in Materials and Methods, and the results are presented in Tables 11 and 12. The data were not corrected for recoveries of the labile minor nucleosides since quantitative comparisons amongst the isoacceptors were deemed sufficient for this analysis. The 2'-O-methyl-nucleosides are not detected in this analysis; however, identical fingerprints were obtained from each of the purified tRNAPhe isoacceptors after RNase T$_1$ digestion and subsequent two-dimensional electrophoresis (24, 25), thus eliminating a 2'-O-methylguanosine deficiency as the lesion in *mia* tRNAs (results not shown). Since 2'-O-methylcytidine is not present in mature tRNATyr (23), which is also affected by *mia* (15), this eliminates it as the lesion in *mia* tRNAs. A representative fluorogram of the separated nucleoside trialcohol derivatives from the purified wild type tRNAPhe of *MIA*$^+$ and each tRNAPhe isoacceptor from *mia* is presented in Fig. 14.

It can be seen from Table 11 that the most significant difference amongst the nucleoside compositions of the

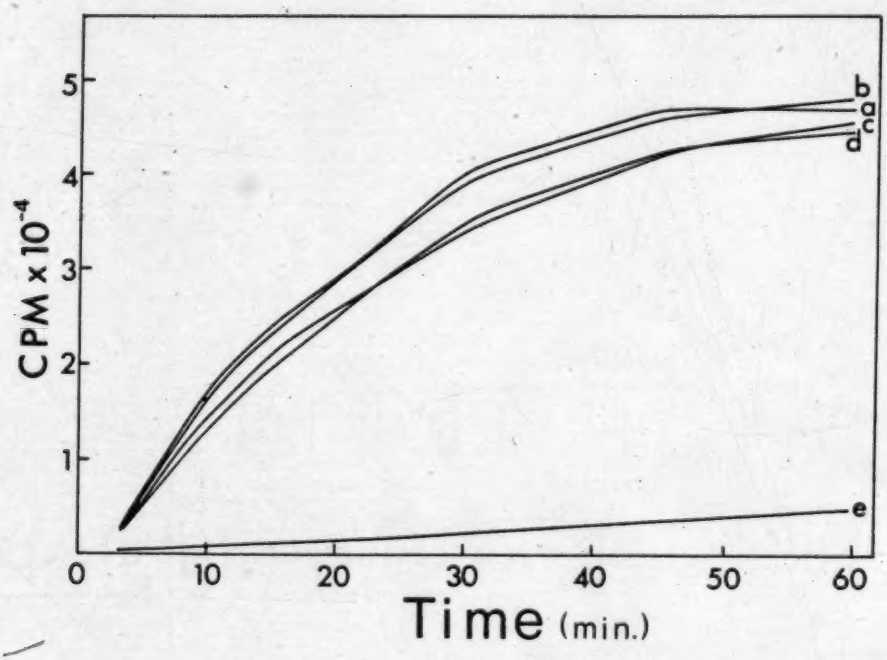


Figure 13

In vitro kinetics of poly-phenylalanine production using each purified $tRNA^{Phe}$ isoacceptor independently in the wheat-germ translation assays. ~0.1 µg of each purified $tRNA^{Phe}$ sample were used: a) wild type, b) isoacceptor A, c) isoacceptor B, d) isoacceptor C, e) no addition of tRNA.

Table 11. Nucleoside compositions of the purified $tRNA^{Phe}$ isoacceptors*

* Relative molar amounts of the nucleosides calculated according to equation 3 in Randerath *et al.* (21). The average recovered cpm /chromatogram is 9×10^5.

+ See reference 23.

‡ The 2'-O-methyl-nucleosides can not be detected by this analysis.

§ All values are averages of three independent determinations with an average coefficient of variation of 11.58 ± 9.4.

¶ No detectable amounts were obtained.

Nucleoside	Expected[+,‡]	*mia*[+]	*mia* isoacceptor A	*mia* isoacceptor B	*mia* isoacceptor C
A	17	14.5[§]	14.7	15.0	14.1
U	12	14.0	14.4	15.0	16.5
G	18	16.7	18.5	17.9	15.8
C	15	17.8	17.4	17.2	16.9
m^2G	1	0.58	0.63	0.52	0.54
m_2^2G	1	1.66	1.13	1.28	2.07
m^7G	1	0.79	0.76	0.74	0.80
m^5C	2	1.30	1.33	1.27	1.41
m^1A	1	0.46	0.42	0.49	0.43
Y	1	0.48	0.51	0.58	0.55
T	1	1.00	0.85	0.87	0.97
Ψ	2	2.82	2.28	2.44	3.77
D	2	1.67	1.32	0.65	0-[¶]
U+D	14	15.7	15.7	15.6	16.5
U+D+Ψ	16	18.5	18.0	18.1	20.3

Table 12. Nucleoside compositions of the purified $tRNA^{Tyr}$ isoacceptors

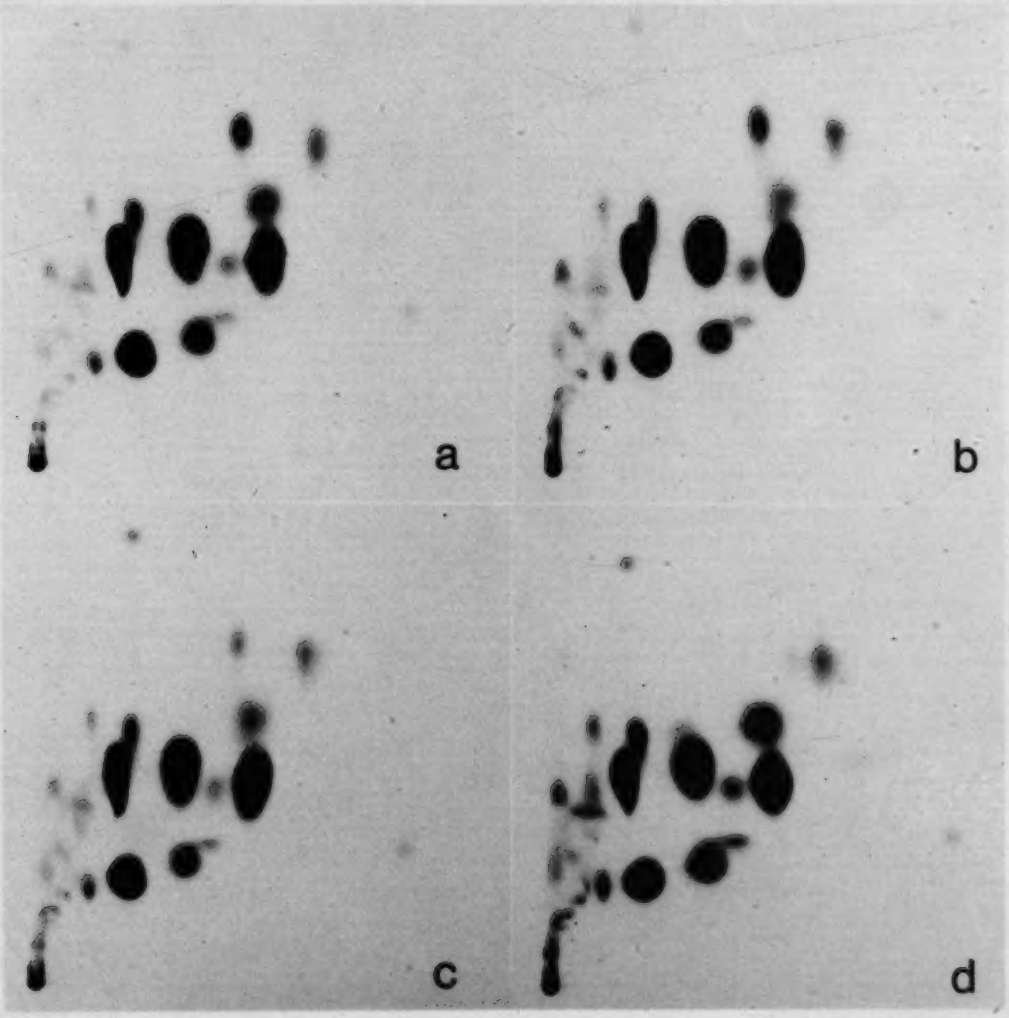
Nucleoside	Expected[+,‡]	*mia*[+]	*mia* isoacceptor D
A	15	12.8[§]	13.1
U	7	10.2	13.9
G	20	21.1	21.0
C	20	22.1	21.1
m^2G	1	0.55	0.45
m_2^2G	1	1.07	-[¶]
m^5C	1	0.57	0.42
m^1A	1	0.51	0.42
i^6A	1	0.34	0.29
T	1	0.87	0.79
Ψ	3	2.56	2.35
D	6	4.48	1.96

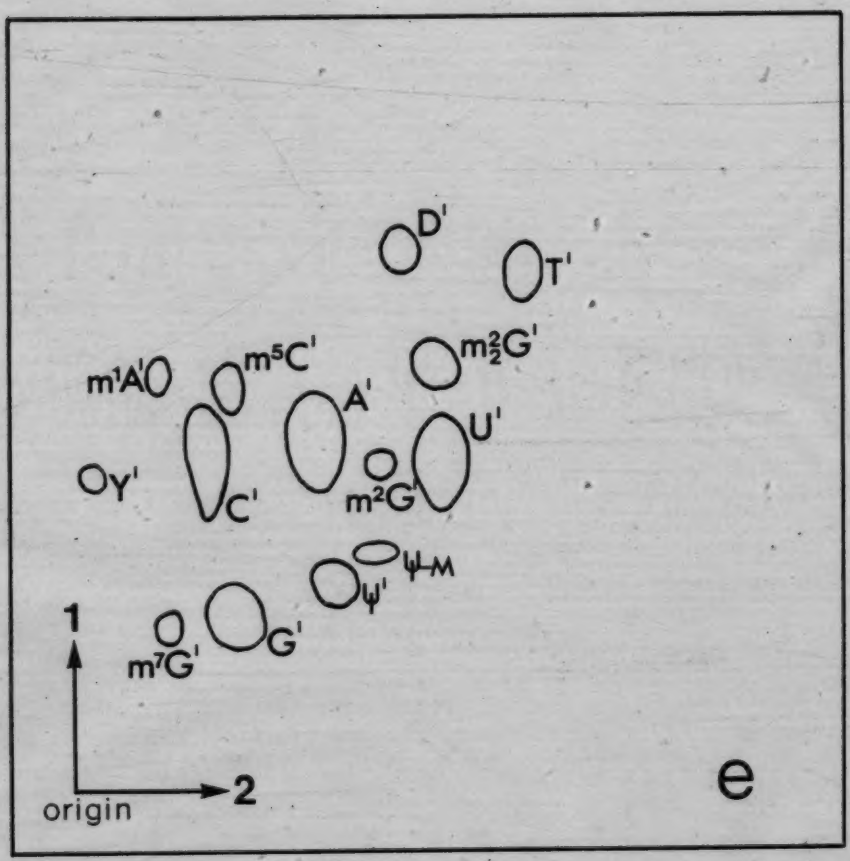
*, +, ‡, § - As in Table 11, except that the average recovered cpm/chromatogram is 7.3×10^5.

¶ - Not determined due to the presence of contaminating materials over that nucleoside.

Figure 14

Nucleoside composition analyses by two-dimensional thin layer chromatography of nucleoside trialcohols from the purified $tRNA^{Phe}$ isoacceptors. The fluorograms were developed as described in Materials and Methods: a) wild type, b) isoacceptor A, c) isoacceptor B, d) isoacceptor C, and e) a diagramatic description of the fluorograms and identification of the nucleoside derivatives. Ψ-M is a monoaldehyde derived from Ψ (21). See Table 11 for quantitative measurements.


a
b
c
d


D'
T'
m¹A'
m⁵C'
m²₂G'
A'
U'
Y'
C'
m²G'
ψ-M
ψ'
1
m⁷G'
G'
origin
2
e

tRNAPhe isoacceptors is in the molar amounts of dihydrouridine, which agrees with the impression one gets from Fig. 14. Transfer RNAPhe from *MIA*$^+$ and isoacceptor A from *mia* are essentially identical in their nucleoside composition, which is consistent with previous observations that isoacceptor A is wild type tRNAPhe (13). Isoacceptor B differs from isoacceptor A or wild type tRNAPhe in having only half the amount of dihydrouridine, while isoacceptor C does not have any detectable dihydrouridine. There are two molecules of dihydrouridine in wild type tRNAPhe (23) and it appears that *mia* is defective in the production of some of the dihydrouridine moieties on tRNA molecules.

The data from the tRNATyr isoacceptors support this hypothesis. There are six molecules of dihydrouridine in wild type tRNATyr (23) and the data in Table 12 indicate that isoacceptor D of *mia* tRNATyr has only half as many dihydrouridine molecules as wild type tRNATyr. Since there are four isoacceptors of tRNATyr in *mia* and the first isoacceptor is wild type (14), we expect the fourth isoacceptor to have only three dihydrouridine modifications if each isoacceptor is produced by the cumulative defect of lacking an additional dihydrouridine modification.

Another nucleoside that seems to vary in amount amongst the isoacceptors is pseudouridine. Isoacceptor C of tRNAPhe appears to have an increased amount of pseudouridine. The data regarding uridine, dihydrouridine, and pseudouridine for the tRNAPhe isoacceptors were further analysed in Table

11. It can be seen that the total amount of uridine and dihydrouridine remains the same amongst the tRNAPhe isoacceptors, while the total amount of uridine, dihydrouridine, and pseudouridine is slightly higher for isoacceptor C. Since dihydrouridine and pseudouridine are derivatives of uridine, this suggests that the increased amount of pseudouridine in isoacceptor C is probably due to contaminants not originating from the tRNAPhe molecules.

Discussion

Purified tRNA isoacceptors from *mia* are probably the same size as cognate tRNAs from wild type yeast and, furthermore, no differences can be detected in the abilities of individual tRNAPhe isoacceptors to participate in *in vitro* translation as measured by the production of poly-phenylalanine. These results support our previous supposition (15) that the mutant isoacceptors are biologically functional.

Comparisons of the nucleoside compositions of the purified tRNAPhe isoacceptors indicate that the mutant isoacceptors have a reduced amount of dihydrouridine compared to wild type tRNAPhe. This analysis identifies the lesion causing the mutant acceptors, and the results are consistent with the hypothesis that *mia* is a leaky mutation resulting in incomplete conversion of the appropriate uridines to dihydrouridines. Previous results (15) which suggested a precursor-product relationship between mutant

acceptors and wild type tRNA can be explained by the present data. Two dihydrouridine moieties are found in mature wild type tRNA (23) and *mia* isoacceptor A; isoacceptors B and C are accumulated, and identified, due to the defect of lacking one or two dihydrouridine modifications, respectively. The undermodified tRNA molecules are still substrates for the defective modification enzyme, which slowly converts them to mature molecules (15). However, the conversion is never complete, so either the enzyme is not active at the later stages of a cell culture, or the tRNA molecules are no longer suitable substrates if they are not modified at the proper time. In the case of the tRNATyr isoacceptor D, the data indicate that it is probably missing three dihydrouridine moieties.

Previous attempts to identify a particular modification deficiency in *mia* strains by examining the primary sequences of yeast tRNAs (23) and comparing these to the pleiotropic array of mutant acceptors found in *mia* (13, 15) failed to implicate dihydrouridine, since it is present in all yeast tRNAs and not all are affected by *mia*. Furthermore, it has been reported (6) that tRNAs differing only in uridine versus dihydrouridine content can not be resolved by chromatography of the intact molecules and, since *mia* tRNAs are routinely identified on RPC-5 columns, this also discouraged us from considering a deficiency in dihydrouridine content as the molecular basis for the mutant forms of *mia* tRNAs.

. Now that it has been shown that the primary effect of *mia* is a deficiency in dihydrouridine formation, a remaining question is why are only some of the tRNAs affected by the mutation. It is possible that there is a separate enzyme for dihydrouridine formation in the subset of tRNAs not affected by *mia* , which could also be responsible for some of the dihydrouridine modifications in those tRNAs which are affected by *mia* ; e.g., in tRNATyr from *mia* only three mutant forms are observed (14) and the mature molecule contains six dihydrouridines (23). There is a paucity of data on the formation of dihydrouridine in all organisms (6). However, there are at least two enzymes for pseudouridine formation in the tRNAs of *Salmonella* (9) and 1-methyl-guanosine formation in yeast tRNAs (26). These examples are precedents for the involvement of more than one enzyme in the production of a particular type of nucleoside modification. Alternatively, the leaky nature of this *mia* mutation could account for the observation that some tRNAs are never affected, if we assume that the defective enzyme preferentially modifies a subset of tRNAs.

We have attempted to obtain mutant tRNA profiles for those tRNAs known not to be affected by *mia* (e.g., tRNAGly) by examining tRNAs from *mia* strains grown under conditions where virtually no wild type tRNAPhe is produced (15). These results show that the tRNAGly profile is still wild type (data not shown). Moreover, the tRNATyr profile in this case has demonstrably higher proportions of mutant isoacceptors

but no new mutant acceptors are evident (i.e., only three mutant forms appear but they represent a much higher proportion of total tRNATyr). Therefore, although it is still an open question as to whether more than one enzyme is involved in dihydrouridine formation in yeast tRNA, our results favor this hypothesis.

Further experiments with *mia* could address the problem of determining the function of dihydrouridine in tRNA since this is presently unknown (6, 7). If *mia* were grown under conditions favouring the maximum production of dihydrouridine deficient tRNAs, the yeast cells could then be examined to ascertain whether or not any particular cellular functions, in which tRNAs are known to be involved (27), are impaired. The existence of a species of tRNA (isoacceptor C of *mia* tRNAPhe) completely devoid of dihydrouridine also provides a promising substrate for studying the enzymology of dihydrouridine biosynthesis.

Acknowledgements

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VI. Effect of *mia* on the activity of *SUP4*

Introduction

The presence of particular modified nucleosides at specific positions on tRNA molecules suggests that they probably play an important role in tRNA function although contradictory data have been reported on this possibility (Nishimura, 1978). In order to ascertain the function(s) of these modified nucleosides, it should be useful to examine the activities of tRNA molecules lacking particular modifications. The primary function of tRNA is in the translation of mRNA into polypeptides; any alterations in the activities of tRNA molecules in this process may be reflected in the growth rates of the cells. In *E. coli* , a mutant that lacks ribothymidine in its tRNAs appears to grow normally on its own, but is out-competed by wild type cells when both are grown together (Björk and Neidhardt, 1975). However, due to the multiplicity of tRNA genes in the eukaryotic cell and the presence of many isoaccepting tRNAs, subtle changes may be difficult to detect when comparing the growth kinetics of modification defective mutants with wild type strains. In fact, none of the modification defective mutations in *S. cerevisiae* seem to affect the growth rate of cells harbouring the mutations (Phillips and Kjellin-Straby, 1967; Laten *et al.*, 1978; A. Hopper, personal communication and this study).

A more sensitive method of studying the activity of tRNA than simple growth kinetics is to make growth dependent on the functioning of a particular tRNA, namely, by examining the efficiencies of informational suppressors. In these studies, the translational activity of a particular tRNA species (the suppressor) in the suppression of certain nonsense mutations is examined under conditions requiring suppression for the survival of the cells. Suppressor efficiency will be reflected in the growth kinetics of mutants containing specific suppressible alleles. As an example, the *mod5-1* mutation of *S. cerevisiae*, defective in the production of isopentenyladenosine (i⁶A) was isolated as a mutation which decreases the activity of the ochre suppressor *SUP7-1* on the ochre mutations *ade2-1* and *can1-100*, but does not have any significant affects on *SUP*⁺ cells (Laten *et al.*, 1978).

It has been suggested that the translational activity of a tRNA at a particular codon is influenced by the mRNA sequence adjacent to that codon; this has been described as the context effect on translation (Salser, 1969; Akaboshi *et al.*, 1976; Feinstein and Altman, 1977; Fluck *et al.*, 1977; Feinstein and Altman, 1979; Engelberg-Kulka, 1981). Recently, it has been demonstrated that the effect of an amber suppressor in translating the UAG codon can vary over an order of magnitude depending on the nucleoside sequence adjacent to the 3' side of the codon (Bossi and Roth, 1980). One explanation suggested to explain these effects is that

neighbouring tRNA molecules on the ribosome sites interact with each other and this influences the binding of tRNAs at the ribosome sites (Bossi and Roth, 1980). As a result, the translational activity of tRNAs at particular codons may vary significantly. These effects should be kept in mind when examining the activities of suppressors on particular nonsense mutations. Transfer RNAs lacking certain nucleoside modifications may have an altered configuration and may interact differently at the ribosome sites, thus also affecting the efficiency of translation.

Now that it has been demonstrated that *mia* affects the production of dihydrouridine normally found in tRNAs, it is interesting to determine if the absence of this modification affects the functioning of tRNA. In *Saccharomyces cerevisiae*, there are eight tRNATyr genes located at different loci (Hawthorne and Leupold, 1974; Olson *et al.*, 1977) and all code for the same mature tRNA (Madison and Kung, 1967). The tRNATyr genes have been identified with each of the eight tyrosine-inserting suppressors (Sherman *et al.*, 1973; Olson *et al.*, 1977). The sequence of three wild type tRNATyr genes and one of the tyrosine-inserting ochre suppressor genes, *SUP4* , has been determined (Goodman *et al.*, 1977). The *SUP4* gene differs from the wild type genes by a single base substitution at the anticodon position which allows the suppressor tRNA to read the ochre codon UAA. Since tRNATyr is one of the tRNA species affected by *mia* , it is possible to study the activities of *mia SUP4*

double mutants on the suppression of ochre mutations. Strains carrying *mia* (or *MIA*$^+$), *SUP4* and the ochre mutations *ade2-1*, *lys2-1*, *met4-1*, *can1-100* were constructed. The translational activity of *SUP4* was analysed by examining the growth kinetics of the yeast strains on omission media which required the suppression of one of the ochre mutations.

Construction of strains

Strains that carry *mia* (or *MIA*$^+$) *SUP4* and the ochre mutations *ade2-1*, *lys2-1*, *met4-1* and *can1-100* were constructed using the crossing scheme described in Figure 15.

Diploid RL113 was obtained by crossing JB760-1C with J12-9A and this diploid was then sporulated. The genotypes of JB760-1C and J12-9A are presented in Tables 3 and 1 respectively, and also in Figure 15. Random spores which carried *ade2-1*, *lys2-1*, *met4-1* and *can1-100* were isolated and scored for *mia* by extracting the tRNAs from individual spore cultures and analyzing them by RPC-5 chromatography. Spore RL113-2, carrying *mia* and the four ochre mutations, was identified and crossed wth J15-13C. The genotype of J15-13 is presented in Table 1 and in Figure 15. Diploid RL114, a homozygote for *ade2-1*, *lys2-1*, *met4-1* and *can1-100* was isolated by micromanipulation and then sporulated. Dissected tetrads were recovered and scored for the segregation of *SUP4* by the suppression of the ochre

Figure 15

Construction of strains carrying *mia* (or *MIA*$^{+}$), *SUP4*, *ade2-1*, *lys2-1*, *met4-1* and *can1-100*.

Cross RL113

RL760-1C α + + + + *mia* +

X

J12-9A *a* *ade2-1* *lys2-1* *met4-1* *can1-100* + +

↓

diploid RL113

↓ isolate random spore RL113-2

Cross RL114

RL113-2 *a* *ade2-1* *lys2-1* *met4-1* *can1-100* *mia* +

X

J15-13C α *ade2-1* *lys2-1* *met4-1* *can1-100* + *SUP4*

↓

diploid RL114

↓ dissect tetrads

Tetrads RL114 -

A*	*a*	*ade2-1*	*lys2-1*	*met4-1*	*can1-100*	+	*SUP4*
B	α	*ade2-1*	*lys2-1*	*met4-1*	*can1-100*	ND	+
C*	α	*ade2-1*	*lys2-1*	*met4-1*	*can1-100*	*mia*	*SUP4*
D	*a*	*ade2-1*	*lys2-1*	*met4-1*	*can1-100*	ND	+

Only the relevent genotypes of the strains are presented

ND - not determined

* - sister spores A and C were used for further experiments

mutations. The presence of *mia* in the *SUP4* spores was again scored for by RPC-5 chromatography of relevant aminoacyl-tRNAs. Sisters spores with the proper genotypes were used for further experiments.

Results

Three pairs of sister spores from the crossing scheme described in Figure 15 were used in the growth kinetics studies. They are RL114-2A *MIA*$^+$, RL114-2C mia ; RL114-3A *mia* , RL114-3B *MIA*$^+$ and RL114-7A *mia* , RL114-7C *MIA*$^+$; all carrying *SUP4* and the ochre mutations *ade2-1*, *lys2-1*, *met4-1* and *can1-100* . The growth kinetics studies were carried out as described in Materials and Methods. Since it is known that the expression of *mia* is influenced by temperature, growth kinetics were examined from cultures grown at 23°, 30° and 37°C.

The growth kinetics of RL114-7A *mia* and RL114-7C *MIA*$^+$ at 23° and 30°C in the different types of media are presented in Figures 16 and 17. The data for the suppression of *can1-100* are not presented since no growth was observed for either strain at any temperature. This is probably a result of the suppression of *can1-100* by *SUP4* , thus making the cells sensitive to canavanine. Further, RL114-7A *mia* does not appear to grow at 37°C in synthetic complete while RL114-7C *MIA*$^+$ grows with similar kinetics as that of 23°C. The data in Figures 16 and 17 suggest that at 30°C, *SUP4* does not appear to suppress *lys2-1* as well with *mia* present,

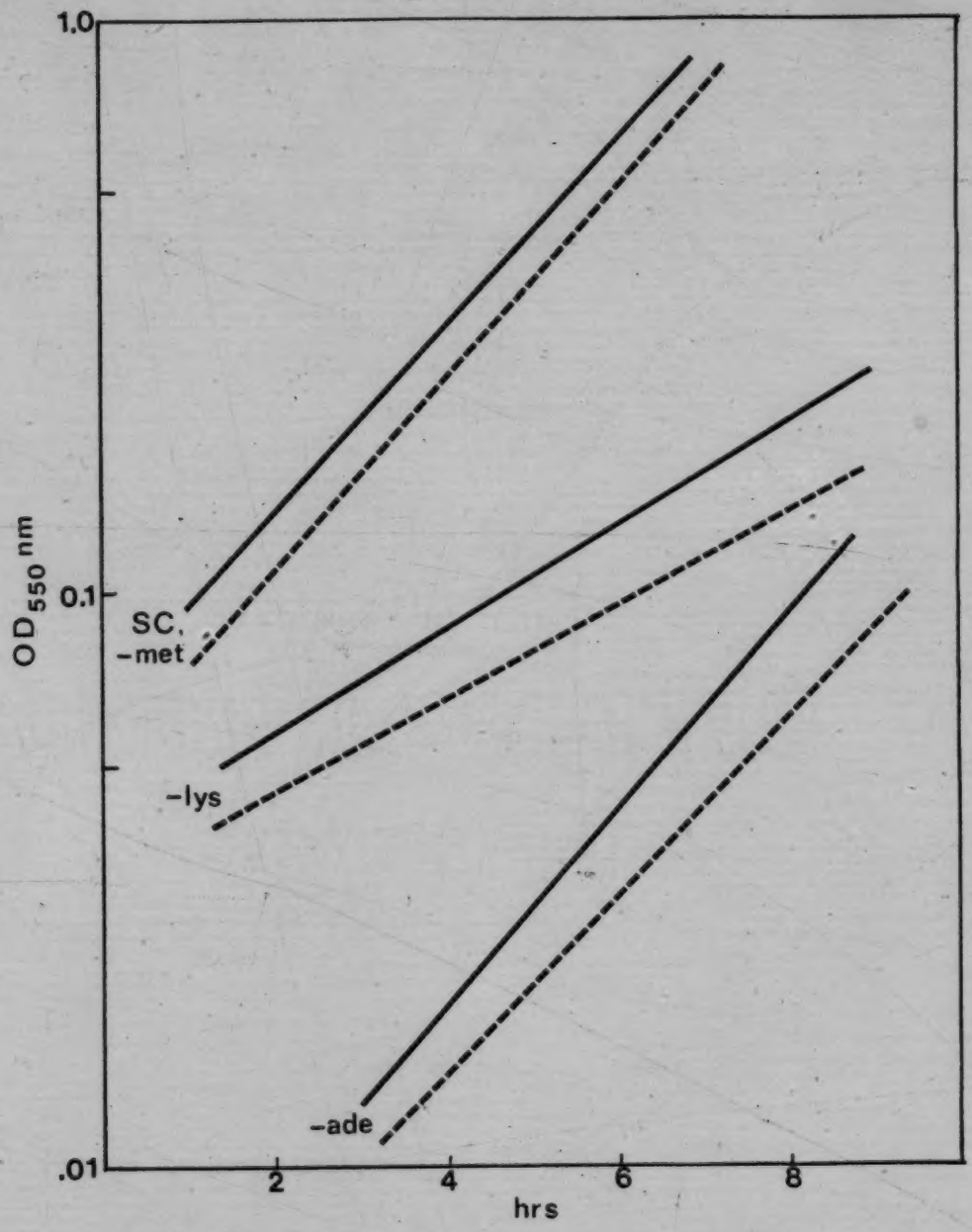

Figure 16

Growth kinetics of RL114-7A *SUP4 mia* and RL114-7C *SUP4 MIA*$^+$ in synthetic complete, methionine omission, lysine omission and adenine omission media at 23^{o}C.

----- RL114-7A *SUP4 mia*

——— RL114-7C *SUP4 MIA*$^+$

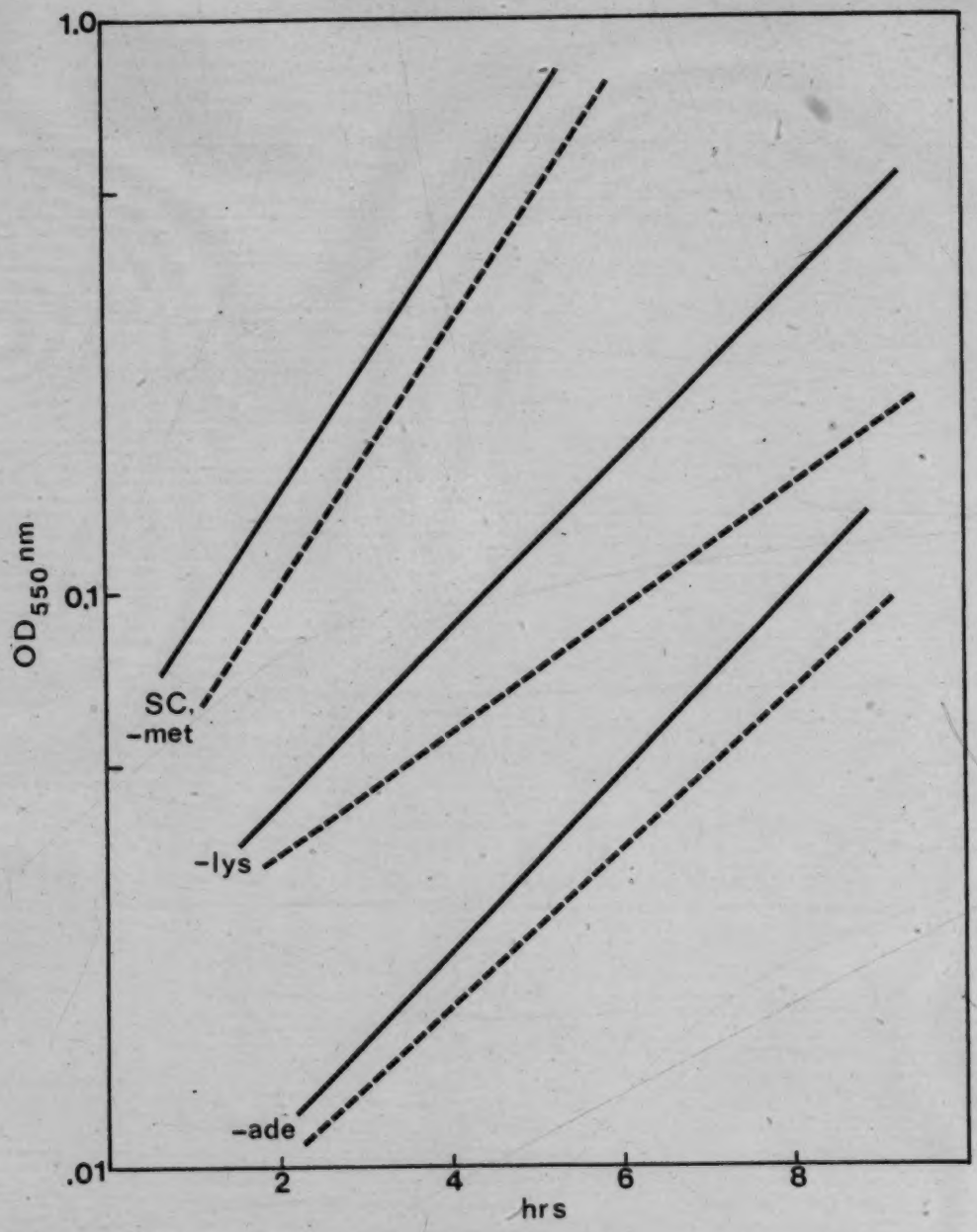

Figure 17

Growth kinetics of RL114-7A *SUP4 mia* and RL114-7C *SUP4 MIA*$^+$ in synthetic complete, methionine omission, lysine omission and adenine omission media at 30^{o}C.

----- RL114-7A *SUP4 mia*

——— RL114-7C *SUP4 MIA*$^+$

as with *MIA*$^+$ present, while there does not appear to be any difference at 23°C. Since *mia* is not expresed at 23°C, these results are consistent with *mia* affecting the activity of *SUP4* at 30°C.

In order to confirm the above observations, the growth kinetics of RL114-2A *MIA*$^+$, RL114-2C *mia* and RL114-3A *mia*, RL114-3B *MIA*$^+$ in synthetic complete and lysine omission media at 30°C were also determined. The results are presented in Figures 18 and 19. The results for RL114-2A *MIA*$^+$ and RL114-2C *mia* (Fig. 18) are consistent with those observed for RL114-7A *mia* and RL114-7C *MIA*$^+$ in that *mia* appears to decrease the activity of *SUP4* on *lys2-1* . However, the results for RL114-3A *mia* and RL114-3B *MIA*$^+$ (Fig. 19) are not in agreement with the other data. In this case, there are no differences in the growth kinetics between the two strains. This indicates that the apparent differences observed in Figures 17 and 18 for the suppression of *lys2-1* are not due to *mia*, but rather are probably due to genetic background differences amongst the strains.

The growth kinetics of RL114-2A *MIA*$^+$, RL114-2C *mia* and RL114-3A *mia* , RL114-3B *MIA*$^+$ at 37°C in synthetic complete and lysine omission media were also determined. The results indicate that the apparent failure of RL114-7A *mia* to grow at 37°C is also not due to *mia* since RL114-3A *mia* grows better than RL114-3B *MIA*$^+$ (data not shown).

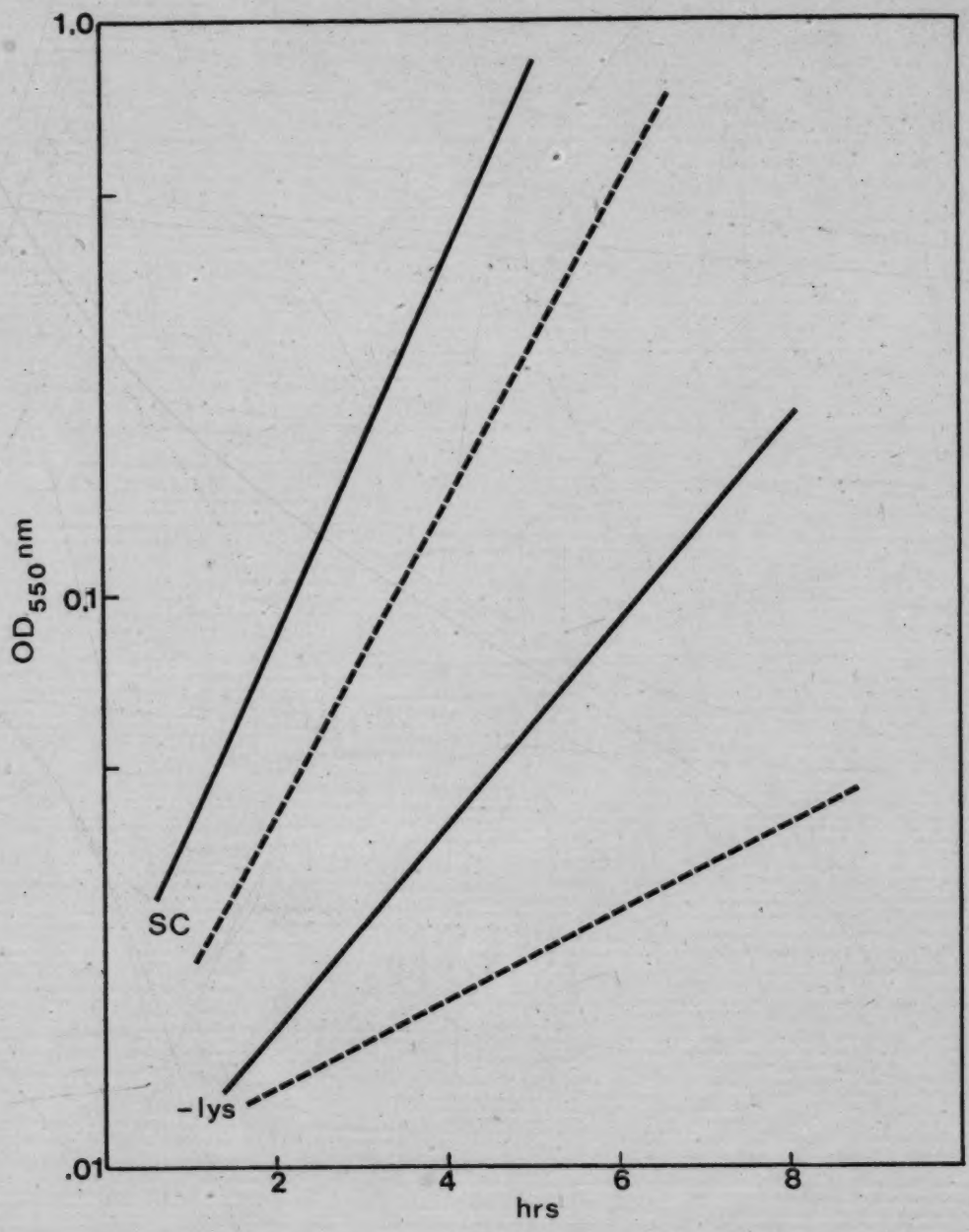

Figure 18

Growth kinetics of RL114-2A *SUP4 MIA*$^+$ and RL114-2C *SUP4 mia* in synthetic complete and lysine omission media at 30^{o}C.

——— RL114-2A *SUP4 MIA*$^+$

----- RL114-2C *SUP4 mia*

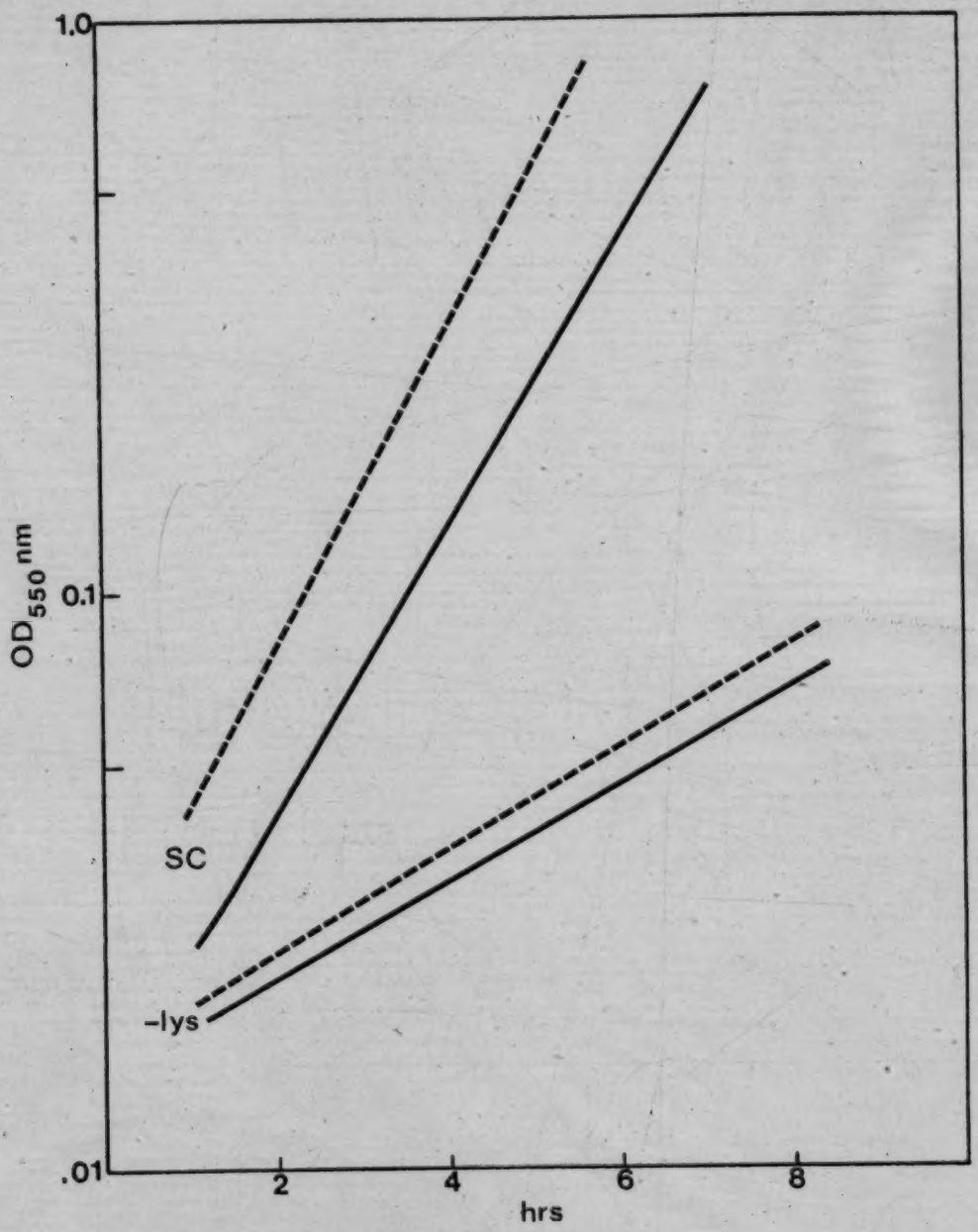

Figure 19

Growth kinetics of RL114-3A *SUP4 mia* and RL114-3B *SUP4 MIA*$^+$ in synthetic complete and lysine omission media at 30^{o}C.

----- RL114-3A *Sup4 mia*

——— RL114-3B *Sup4 MIA*$^+$

Discussion

The results presented indicate that *mia* does not seem to have any significant effects on the translational activity of *SUP4* on a number of ochre mutations. Some differences were observed for RL114-7A *MIA*$^+$ and RL114-7C *mia* in the suppression of *lys2-1* (Fig. 17), but apparently this is not an effect of *mia* since RL114-3A *mia* and RL114-3B *MIA*$^+$ (Fig. 19) show similar suppression efficiencies for *lys2-1*. The failure of the strains RL114-7C *mia* , RL114-2C *mia* and RL114-3B *MIA*$^+$ to grow on synthetic complete media at 37°C indicate that there are genetic differences other than *mia* amongst the strains which affects their growth rates. These background differences do not seem to affect the growth rates of cells at the lower temperatures, as indicated by the parallel growth curves in synthetic complete.

An earlier attempt to detect any effect of *mia* on *SUP4* by a petri-plate assay also failed to detect any significant differences between the same pairs of spores at 30°C (data not shown). It has also been observed that the *trm1* mutation does not seem to affect the activity of *SUP4* (A. Hopper, personal communication). So neither dihydrouridine nor N^2-dimethylguanosine seem to be required for the suppressor activity of tRNA. On the other hand, i^{6}A affects the activity of the suppressor tRNA since the *mod5-1* mutant, defective in i^{6}A modification, was isolated by the reduction of the activity of the ochre suppresor *SUP7-1* (Laten *et al.*, 1978). The locations of these three modifications on tRNA

differs from each other in that i⁶A is normally found next to the anticodon, while dihydrouridine and N^2-dimethylguanosine are normally found primarily on the D loop and between the D-stem and the anticodon-stem respectively (see Fig. 1). It was suggested that the i⁶A deficiency affects tRNAs in protein synthesis at a step after aminoacylation (Laten *et al.*, 1978). It is possible that i⁶A is involved in stabilizing the base pairing between codon and anticodon in the suppressor tRNA. The locations of dihydrouridine and N^2-dimethylguanosine do not immediately imply any involvement in codon-anticodon interactions and therefore a negative result may not be surprising from the suppressor assays. The data on *mia* or *trm1* tRNAs indicate that each can also be aminoacylated (see Chapter 4). It is still very possible that dihydrouridine and N^2-dimethylguanosine are involved in other biological activities of tRNA which are not detectable in the suppressor assays.

It may be interesting to study the activity of *SUP4* in tRNAs missing both dihydrouridine and N^2-dimethylguanosine even though the outcome may also prove to be uninformative. It is known that strains carrying both *mia* and *trm1* (RL101-5B) grow as well as wild type and that tRNAs missing both dihydrouridine(s) and N^2-dimethylguanosine can still be aminoacylated (see Chapter 4). This suggests that tRNAs missing both types of modifications are still functional in protein synthesis, but it is not known whether the double

mutant may exert any effects on the translational activity of *SUP4* .

VII. Conclusions

The results from genetic, biochemical and physiological studies on the tRNA isoacceptors produced in the yeast strain XB109-5B have identified and characterized a mutation in *Saccharomyces cerevisiae* which affects the modification of uridine to dihydrouridine in tRNA molecules. These mutant tRNAs, missing certain dihydrouridine modifications, were identified as extra isoaccepting tRNAs by RPC-5 chromatography. Of the nine tRNA species examined from XB109-5B, tRNAPhe, tRNATyr, tRNASer, tRNAVal, tRNAMet and tRNALeu are affected by this mutation in that extra isoaccepting tRNAs are resolved. Transfer RNAAsp, tRNAHis and tRNAGly are not affected and exhibit tRNA profiles identical to that of a wild type strain (S288C).

The genetic basis for the production of these extra (mutant) isoaccepting tRNAs in the several tRNA species was established by segregation analyses. XB109-5B was crossed to wild type (S288C) and the tRNA profiles from the resulting diploids were found to be identical to the wild type profile. Fourteen progeny tetrads were analysed for the segregation of the mutant versus wild type profile of tRNAPhe and all showed a 2:2 segregation pattern. These results suggested that a single recessive mutation is responsible for the production of the mutant tRNAPhe isoacceptors. Further, in one of the progeny tetrads, the

same 2:2 segregation pattern was observed for the other five tRNA species that are known to exhibit mutant profiles. This demonstrated that the same mutation is responsible for the production of the mutant isoacceptors for all the tRNAs observed. This mutation was designated *mia* .

Results from nucleoside composition analyses of the purified tRNAPhe isoacceptors and one of the tRNATyr isoacceptors indicated that the mutant tRNAs are missing some of the dihydrouridine modifications normally present. This identified the actual molecular lesion responsible for the production and original identification of the mutant tRNAs, and suggested that *mia* affects the production of dihydrouridine.

It was observed that the mutant isoacceptors were produced at the expense of the wild type tRNAs. Furthermore, results from the pulse-label and chase experiments demonstrated that the mutant isoacceptors can be converted to the cognate wild type molecules. These results are consistent with the hypothesis that *mia* is affecting a modification process during the maturation of tRNA molecules. Apparently, the mutant isoacceptors can still be modified to the mature tRNAs *in vivo* . This suggested that either the production of dihydrouridine on the tRNA molecules was carried out near the end of the maturation process or that the maturation process does not follow an obligate sequence. Recent reports have demonstrated a sequential order for the nucleoside modifications of yeast

tRNAs, and the modification of dihydrouridine does not appear to be near the end of the maturation process (Melton *et al.*, 1980; Nishikura and De Robertis, 1981). This suggests that the sequential order of nucleoside modifications is not obligatory. The production of mutant tRNAs missing dihydrouridine(s) as well as N^2-dimethylguanosine in *mia trm1* double mutants is consistent with this hypothesis.

There is a paucity of information on the production of dihydrouridine in all organisms. This modification is probably a simple enzymatic reduction of the 5,6 double bond on the uracil moiety and *mia* is probably affecting the actual modification enzyme rather than some other component required for the modification process. This hypothesis is supported by the following observations.

The expression of *mia* was found to be influenced by the growth conditions of the yeast cultures. The *mia* tRNA profiles from cultures grown under non-shaking conditions are the same as wild type. With the increase in shaking and/or aeration of the cultures, the tRNA profiles become more mutant (Bell *et al.*, 1978). Similarly, the production of the mutant isoacceptors is also affected by temperature. The *mia* tRNA profiles from cultures grown with a fast shaking rate at 23°C are also identical to wild type and become more mutant with the increase in temperature. At 37°C, virtually all tRNAs that are affected by *mia* are in the mutant forms. These results are consistent with *mia*

being a leaky mutation affecting the actual modification enzyme. Cultures grown at a fast-shaking rate have a higher amount of dissolved oxygen in the medium due to a higher gaseous exchange rate compared to a non-shaken culture. As a result, the enzymatic reduction of uridine is inhibited and mutant isoaccepting tRNAs lacking dihydrouridine modifications are accumulated. This notion is supported by the observation that *mia* cultures grown at the fast shaking conditons at 30°C but under a nitrogen atmosphere produced very small amounts of the mutant isoacceptors (see Appendices). In the absence of oxygen, even with the fast shaking rate, the defective enzyme was able to carry out the reduction reaction (although at a slower rate) and therefore most of the dihydrouridines on the tRNAs were produced. The temperature effect on the production of the mutant isoacceptors indicates that *mia* is affecting the activity of an enzyme, which is very likely to be the actual modification enzyme. This idea is further strengthened by the observation that *MIA*$^+$ cultures grown at 37°C produced small amounts of mutant isoacceptors. This is probably due to the decreased activity of the enzyme for dihydrouridine production at the elevated temperature.

Examination of tRNA profiles from *mia* cultures at different stages of growth revealed that virtually all *mia* tRNAs during the active growing stages of the cultures are in the mutant forms. This suggested that the mutant isoacceptors missing the dihydrouridine moieties still

function. This is supported by the results from the *in vitro* poly-U translation experiments using the purified tRNAPhe isoacceptors individually in the assays. These results indicate that dihydrouridines are not absolutely required for the functioning of tRNA molecules in protein synthesis. In fact, *mia* strains do not have any obvious morphological phenotypes nor additional nutritional requirements and grow as well as *MIA*$^+$ strains (see Appendices). The mutants in N^2-dimethylguanosine and isopentenyladenosine modifications also do not appear to affect the survival of the cells (Phillips and Kjellin-Stråby, 1967; Laten *et al.*, 1978). This indicates that these modifications are also not necessary for the translational activity of tRNA molecules during protein synthesis although isopentyladenosine deficiency affects the activity of the ochre suppressor *SUP7-1* .

An attempt to detect any effect of *mia* on the efficiency of the ochre suppressor *SUP4* failed to demonstrate any changes in the activity of *SUP4* on a number of ochre mutations. It is possible that dihydrouridine is involved in some other functions of tRNA, but so far there do not seem to be any clear indications. In fact a tRNASer that lacks the dihydrouridine loop and stem completely has been identified from mammalian mitochondria and probably functions as a tRNA in protein synthesis (de Bruijn *et al.*, 1980). This is probably a very special case, but it makes the study of the structure-function relationship of tRNA

molecules a more interesting problem.

To date, there is very little solid evidence which relates the modified nucleosides to any particular function of tRNA molecules. However, the consistency of their appearance suggests that they probably play a part in tRNA function. These modified nucleosides may be involved in some functions of tRNA not yet uncovered. It is hoped that by studying mutants defective in nucleoside modifications, more information about the biosynthesis and functions of the modified nucleosides as well as the mature tRNAs will be accumulated and this may bring about a better understanding of this versatile macromolecule.

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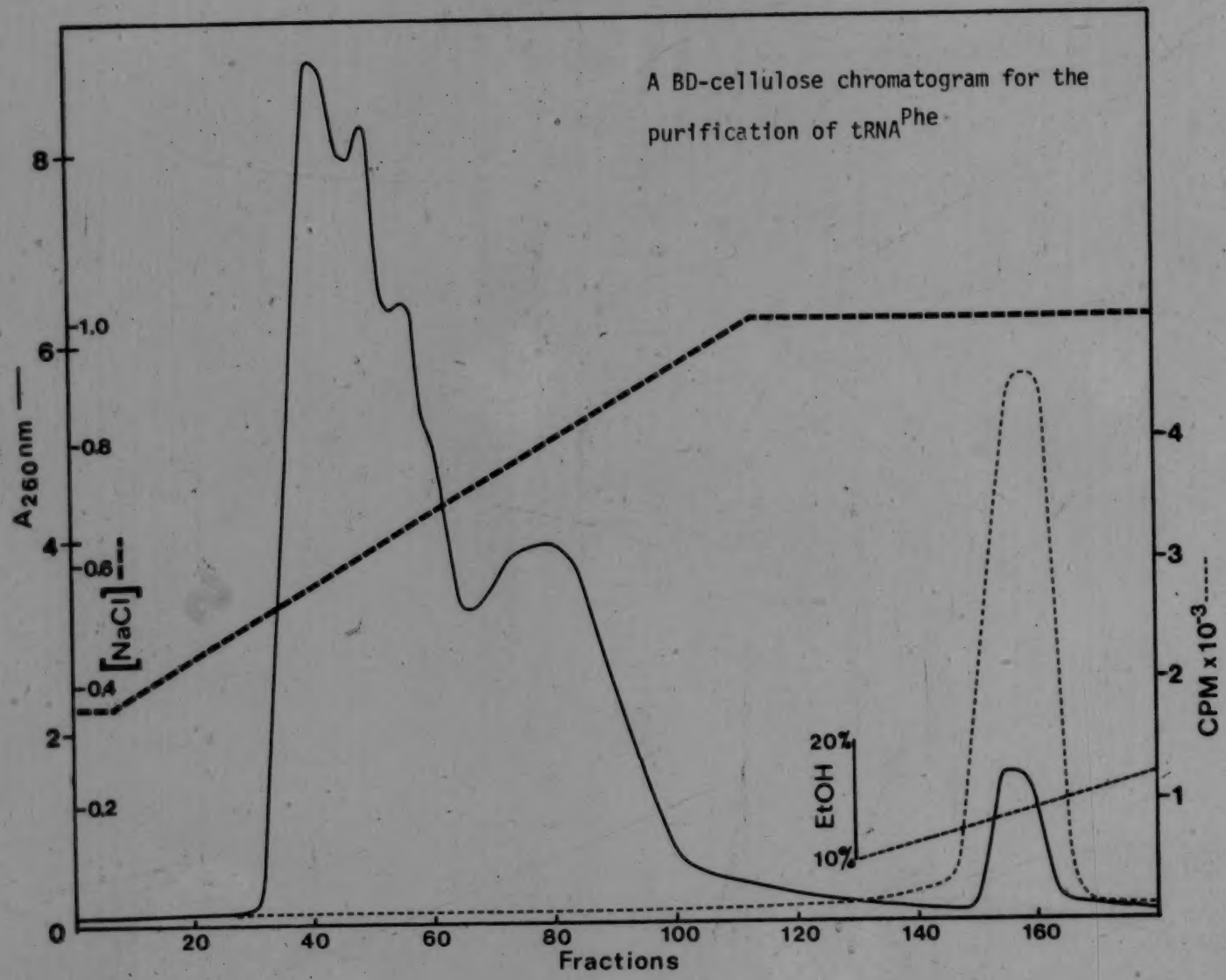
Appendices


A BD-cellulose chromatogram for the purification of tRNA^Phe
A260nm
[NaCl]
CPM x10^-3
EtOH
20%
10%
Fractions
0
2
4
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8
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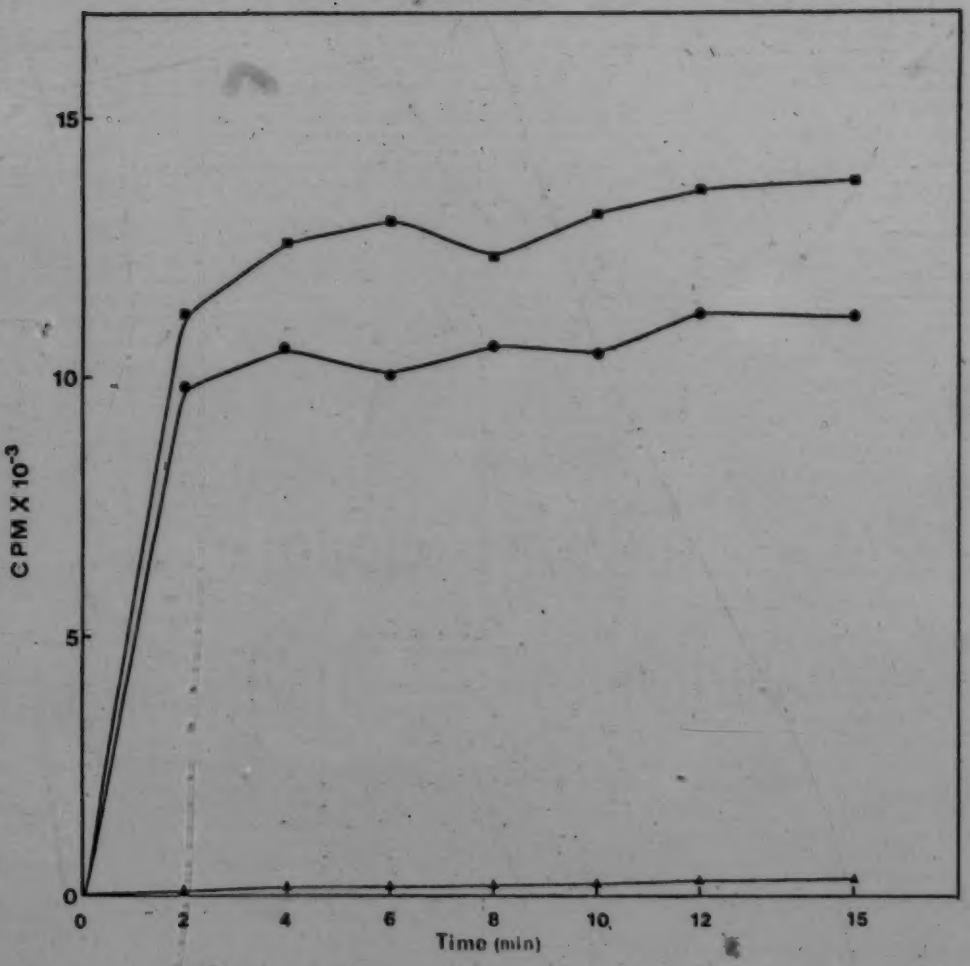
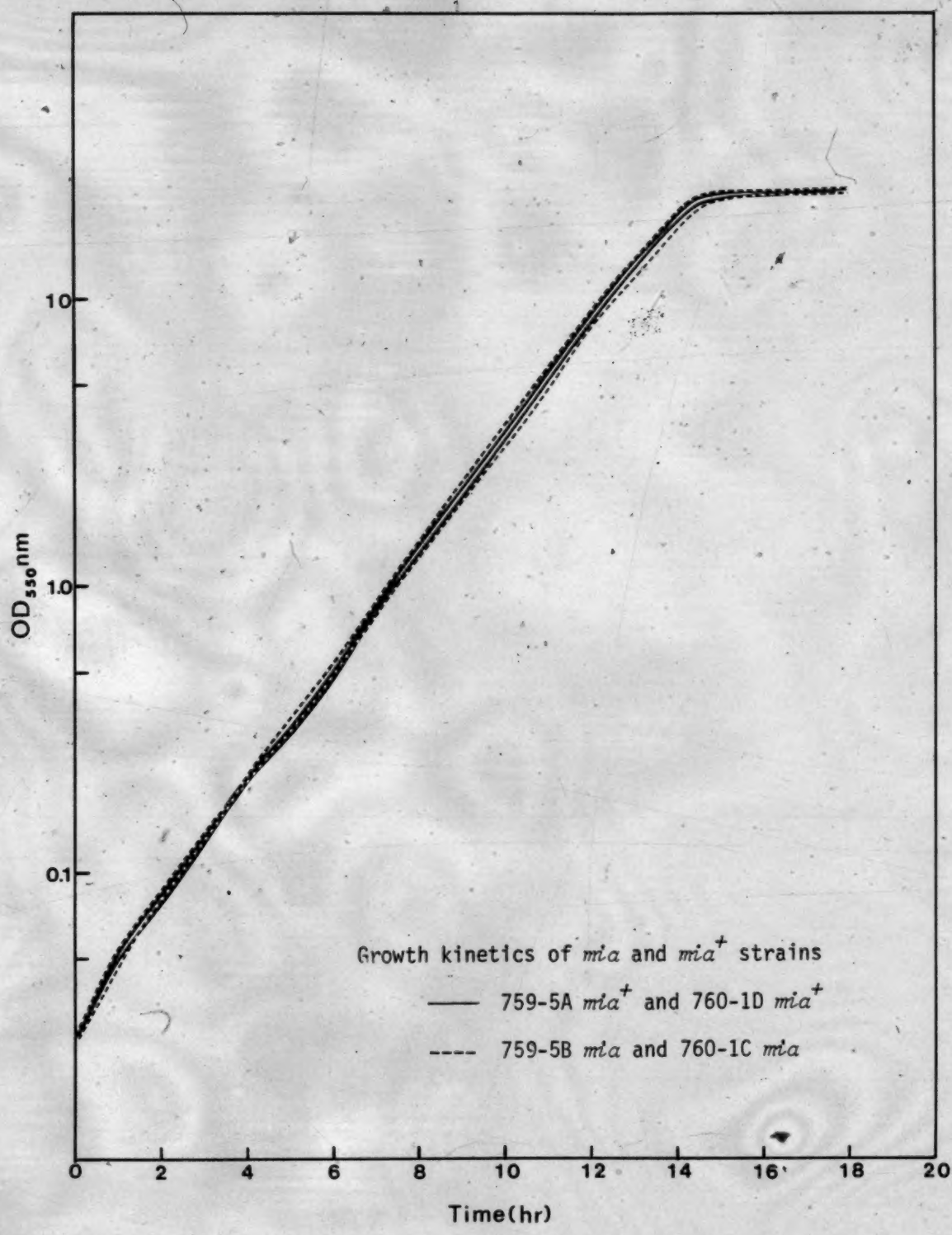


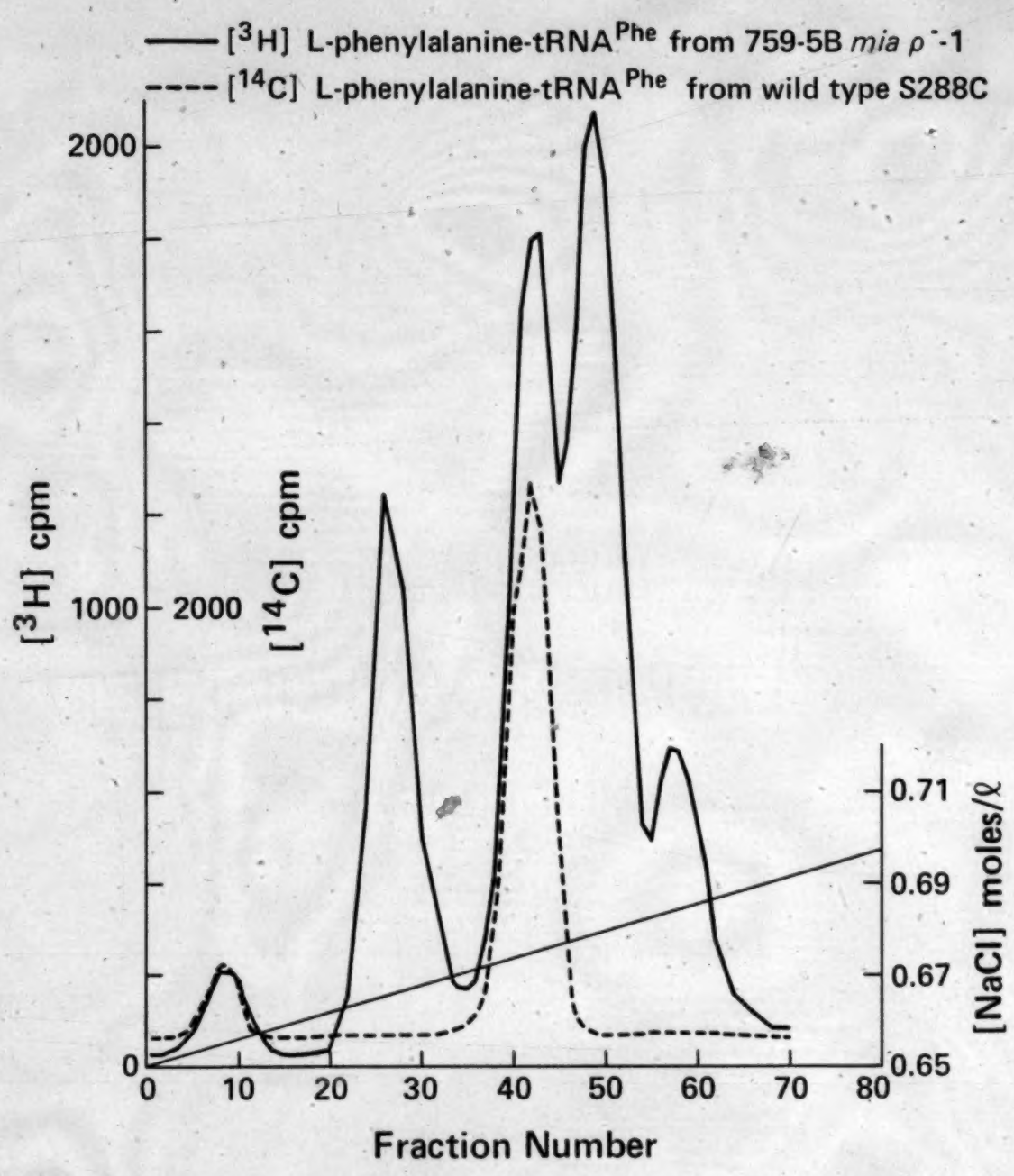
Aminoacylation kinetics of bulk tRNA with [^{14}C] L-phenylalanine

■ - 759-5A *mia*$^{+}$

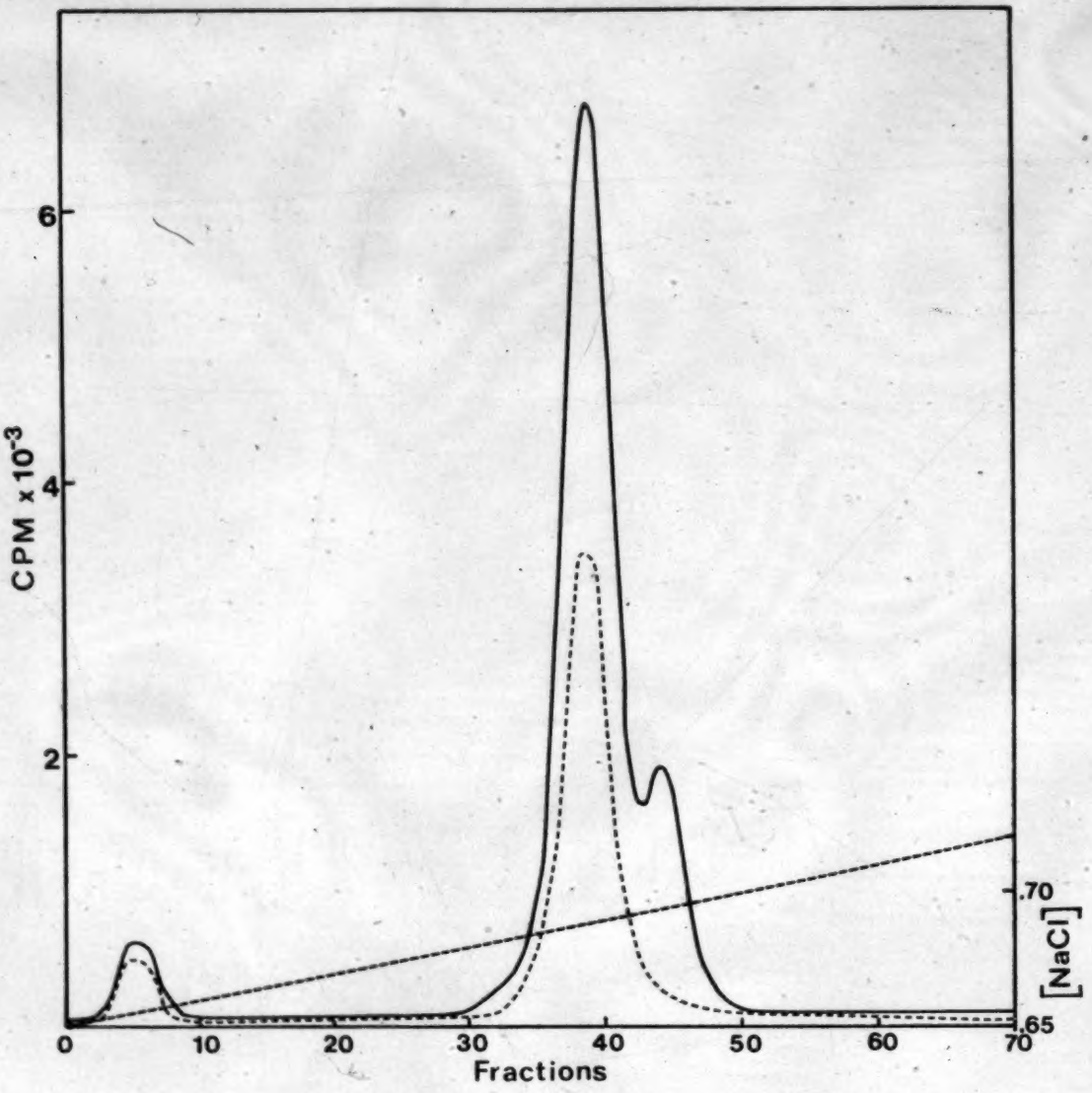
● - 759-5B *mia*

The different levels of saturation is due to differences in the amount of tRNA in each reaction.


1.0
1.0
0.1
OD_{550nm}
0
2
4
6
8
10
12
14
16
18
20
Time(hr)
Growth kinetics of *mia* and *mia*$^+$ strains
—— 759-5A *mia*$^+$ and 760-1D *mia*$^+$
---- 759-5B *mia* and 760-1C *mia*



RPC-5 co-chromatogram of Phe-tRNA^{Phe} from a petite derived from 759-5B *mia* and wild type S288C



RPC-5 co-chromatogram of Phe-tRNA^{Phe} from 759-5B *mia* grown under nitrogen together with control wild type S288C

—— 759-5B *mia* grown under nitrogen

---- wild type S288C